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ON THE NATURE OF THE HYPERTROPHIED PITUITARY GLAND INDUCED IN THE MOUSE BY I^{131} INJECTIONS, AND THE MECHANISM OF ITS DEVELOPMENT

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THE occurrence of pituitary tumors in *mice* treated with radioactive iodine has recently been reported by Gorbman (1949). Pronounced enlargement of the gland was observed as early as 150 days after single injections of 100–1000μc. of I^{131}, and by the time 1–2 years had elapsed, pituitaries as large as 200–240 mg. were found. The normal pituitary in the strains of mice used by Gorbman weighed approximately 2 mg.

In a recent study carried out in this laboratory (Goldberg and Chaikoff, 1950), distinct enlargement of the pituitary gland was not observed in *rats* similarly treated with I^{131}. Rats weighing about 250 gm. were injected with 875μc. of I^{131}. Their glands, examined 11 months later, weighed from 6–12 mg. Pituitary glands of uninjected controls weighed 6–9 mg. The pituitaries of the I^{131}-treated rats were not larger than those observed in rats subjected to surgical thyroidectomy.

Our failure to find pituitary tumors in the rat led us to study the action of I^{131} in the mouse. The enlargement of the pituitary gland in this species was fully confirmed (table 1).

The present investigation deals with the cytological characteristics of the enlarged mouse pituitary induced by I^{131}, and also with the mechanism of its development.

EXPERIMENTAL

Seventy male white mice of the Swiss strain, weighing 25–30 gm., were used in this study. When 6 weeks old, 45 were injected intraperitoneally with 600 μc.[2] of carrier-free

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[1] Atomic Energy Commission Fellow.

[2] The microcurie as used here is based on the Bureau of Standards recalibration of the I^{131} millicurie in effect at Oak Ridge National Laboratories July 1, 1949 (cf. Isotopes Division Circular E-44 from U. S. Atomic Energy Commission).

I^{131}.[3] For the next 3 weeks, these injected mice were fed a basal diet.[4] At the end of that time, the injected mice were divided into two groups. Group B was continued on the basal diet. Group C was fed this same diet, to which had been added 0.15 per cent USP desiccated thyroid.[5] Each mouse of Group C ingested about 2.5 gm. of diet per day, thus ensuring a daily intake of about 2.9 gamma of thyroxine per animal. Twenty-five untreated mice were kept as controls (Group A); these were also fed the basal diet.

All mice were sacrificed between 8 and 11 months of age. They were anesthetized with sodium pentobarbitol, and exsanguinated. The pituitaries were fixed for 12–24 hours in sublimate-formol (9:1). The glands were weighed after initial dehydration in 70 per cent alcohol. They were embedded in paraffin, sectioned at 2 microns, and stained by a modification of Martins' (1933) haematoxylin-trichrome method.

TABLE 1

Group	Number of animals	I^{131} injected	Desiccated thyroid fed with diet	Weight of Pituitary Gland		Cytological characteristics of anterior pituitary
				Range	Mean ± S.D.	
		microcuries	per cent	mg.		
A	25	none	none	2.1– 3.5	2.6±0.3	Normal
B	20	600	none	8.9–18.6	14.9±3.2	Acidophil degranulation, hyperplasia and hypertrophy of basophils; thyroidectomy cells (figs. 1, 3, and 4).
C	25	600	0.15	2.5– 3.5	2.9±0.3	Similar to normal, some glands with increased percentage of basophils (fig. 2).

RESULTS

Gross Examination.—Marked enlargement of the pituitary glands occurred in all mice of Group B, i.e., in those that had been injected with 600μc. of I^{131} and maintained on the basal diet (table 1). The weights of the glands of the 25 normal control mice (Group A) ranged from 2 to 3.5 mg.; those of the I^{131}-injected mice ranged from 9 to 19 mg.

The pituitaries of the mice of Group C did not exceed 3.5 mg. Thus the addition of desiccated thyroid completely prevented the enlargement of the pituitary gland induced by radioactive iodine.

Microscopic Examination.—Group A.—The normal mouse anterior pituitary has been described in detail by Urasov (1927). The three cell types (acidophils, basophils, and chromophobes) are morphologically similar to those found in the anterior pituitary of the rat.

[3] Injections of 200–300 μc. have been found, in this laboratory, to destroy completely the adult mouse thyroid gland. This was shown by serial section of the tracheal region.

[4] The basal diet had the following composition: 68.5 per cent whole wheat, 5 per cent casein, 10 per cent fish meal, 10 per cent alfalfa, 1.5 per cent sodium chloride, and 5 per cent sardilene, a fish oil containing 400 AOAC chick units of vitamin D and 3,000 USP units of vitamin A per gram.

[5] The preparation (Lilly) was stated to contain not less than 0.17 and not more than 0.23 per cent iodine. Dr. Alvin Taurog found it to contain approximately 0.077 per cent thyroxine.

Group B.—No cytological changes were observed in the posterior or intermediate lobes of the gland; the enlargement observed in Group B was confined solely to the anterior portion.

Examination of the anterior pituitaries of the I^{131}-treated mice revealed degranulation of the acidophils and hypertrophy and hyperplasia of the basophils (figs. 1, 3).

The basophilic cells were infrequently found in small clusters, an example of which is shown in Figure 1. These localized areas of basophilic proliferation were not walled off and did not appear to compress adjacent tissue.

The greatly hypertrophied basophils had a finely granular or foamy cytoplasm (fig. 4). Many basophils contained hyaline vacuoles; generally 2–4 small vaculoes were present in each cell (fig. 4). Many basophils contained a single large vacuole, giving rise to the signet ring type cell characteristic of thyroidectomy in other species.

The vesicular round nuclei of the basophils were markedly increased in size (figs. 2–3) and contained one or more fuchsinophilic nucleoli. Polymorphic, lobular, or indented nuclei were not observed.

Considerable mitotic activity was observed in the basophils (fig. 4); no abnormal or atypical figures were seen. There was no cytological evidence of neoplastic change.

Small "colloid" filled cysts, resembling thyroid follicles, were frequently observed. These were usually rimmed by typical basophils (fig. 3). Such cysts can also be found in the normal gland.

Group C.—Cytological examination of the pituitaries of the radioiodine-treated mice *fed desiccated thyroid* revealed a picture (fig. 2) similar to that of the normal controls. Many, however, showed signs of slight thyroxine deficiency (an increased number of basophils and the presence of scattered thyroidectomy cells). The acidophils in a few mice were more numerous and were filled with denser, deeper staining granules than those normally observed.

DISCUSSION

It is shown here that the hypertrophy and the cytological changes induced in the mouse anterior pituitary by the injection of relatively large doses of I^{131} can be prevented by the feeding of desiccated thyroid. This action of desiccated thyroid indicates that the changes produced in the anterior pituitary by the radioactive iodine are secondary to thyroid destruction and not a direct radiation effect of the I^{131} upon the pituitary gland.

Although the cytological changes induced in the mouse anterior pituitary by the I^{131} injections resembled quite closely those already reported

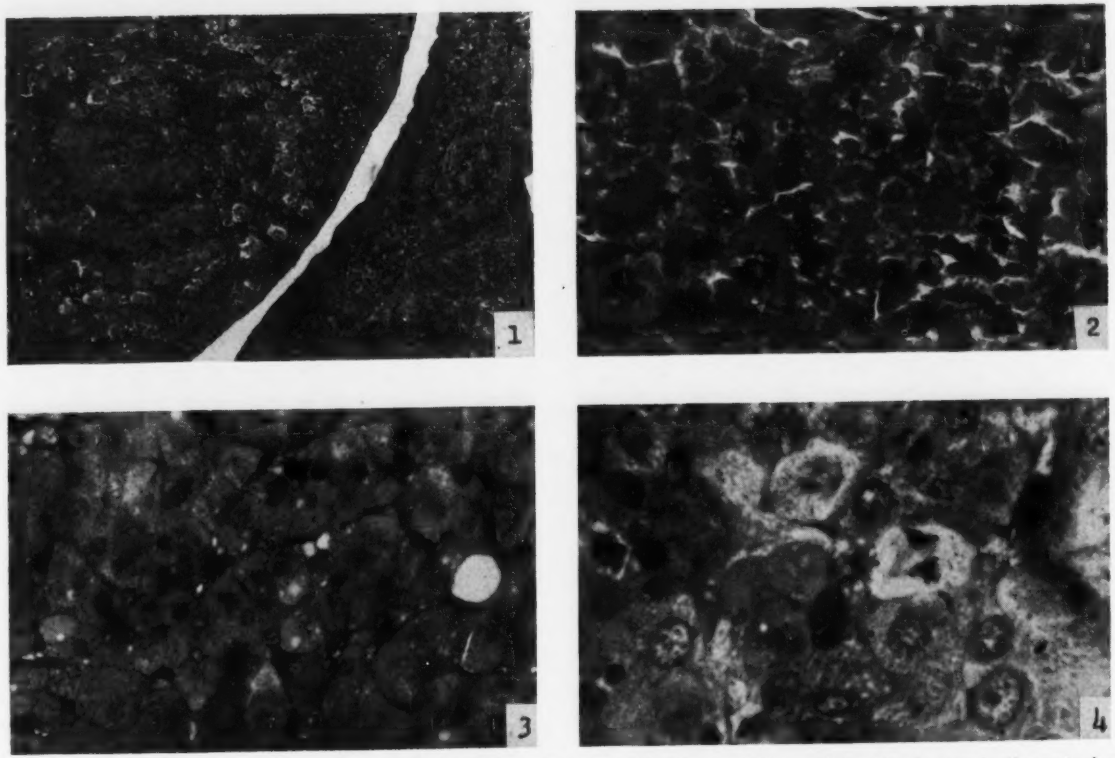

FIG. 1. Pituitary gland of mouse injected with 600 μc. I^{131}. A localized basophilic proliferation is seen in the anterior lobe (to left of cleft). Intermediate and neural lobes are seen to right of cleft. (Martins $\times$100.)

FIG. 2. Anterior pituitary of mouse injected with 600 μc. I^{131} and maintained on 0.15 per cent dessicated thyroid. Note the numerous acidophils (dark cytoplasm) and absence of hypertrophic basophils. (Martins $\times$500.)

FIG. 3. Anterior pituitary of mouse injected with 600 μc. I^{131}. Note absence of acidophils. Hypertrophied and vacuolated basophils completely fill the field. The marked nuclear hypertrophy can be seen by comparison with the nuclei in fig. 2 (same magnification). A basophil-lined, colloid-filled cyst is seen to be right of center. (Martins $\times$500.)

FIG. 4. Hypertrophied basophils from I^{131}-treated mouse. Note vacuolated cell just to the left of center. Mitotic figure of dividing basophil is seen in center of field. (Martins $\times$1,400.)

by us in rats subjected to the same I^{131} treatments, the rat differs from the mouse in showing little, if any, hypertrophy of the adenohypophysis. This was true in rats even when the amounts of I^{131} injected were large anough to destroy completely the thyroid gland (Goldberg, Chaikoff, Lindsay, and Feller, 1950). Recently we have also shown that total destruction of the thyroid gland in the dog by means of I^{131} does not result in significant enlargement of the pituitary gland (unpublished observations).

It should be emphasized that no cytological evidence of neoplastic change was found in the anterior pituitary glands of the I^{131}-treated mice. The longest interval that elapsed between I^{131} injections and examination of pituitaries was 10 months. The possibility that this gland may undergo neoplastic change should not, of course, be ruled out at this time.

Marked enlargement of the pituitary gland was not observed, by Gorbman (1949) in mice fed a diet containing 0.1 per cent thiouracil. The results presented here would seem to indicate that the goitrogen treatment failed to inhibit completely thyroxine synthesis in the thyroid.

SUMMARY

1. The enlargement of the pituitary gland of the mouse treated with I^{131} has been confirmed.

2. It is shown here that this is limited solely to the anterior lobe. Microscopic examination of the anterior lobe revealed a benign hypertrophy and hyperplasia of the basophilic cells. Degranulation of the acidophils also occurs. No cytopathological alterations were observed in the posterior or intermediate lobes.

3. The hypertrophy and cytological changes induced in the anterior lobe by the I^{131} injections were prevented by the feeding of desiccated thyroid. It is concluded that the enlargement resulted from the hypothyroidism which followed the destruction of the thyroid by the ionizing radiation.

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FIG. 4. Hypertrophied basophils from I^{131}-treated mouse. Note vacuolated cell just to the left of center. Mitotic figure of dividing basophil is seen in center of field. (Martins ×1,400.)

ANDROGEN SECRETION IN THE FOWL

ELSIE TABER

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THAT comb growth in the female as well as in the male fowl is controlled by androgen has been well established. Most of the evidence indicates that the interstitial cells, rather than the seminiferous tubules, are the major source of this hormone in the male (Benoit, 1929, 1935a; Nalbandov, Meyer and McShan, 1946; Sluiter and Van Oordt, 1946, 1947). The early work of Domm (1929, 1933) suggested that the medullary component of the left ovary was the main site of androgen production in females, and later cytological studies have shown that the appearance of granular interstitial cells in the medulla seemed to be correlated with male hormone secretion (Benoit, 1935b; Uotila, 1939; Pfeiffer and Kirschbaum, 1943).

It has been demonstrated that vacuolar, foamy-appearing interstitial cells, heavily stained by osmic acid, are characteristic of ovaries and testes from untreated chicks shortly after hatching, but are replaced by cells with more granular cytoplasm at approximately the time comb growth is first apparent. The precocious appearance of granular interstitial cells, as well as precocious comb growth, can be induced by gonadotrophin injections (Taber, 1948a, 1948b, 1949a).

From these results, it seemed likely that the granular interstitial cells were concerned with androgen secretion, while the vacuolar cells represented a relatively inactive phase. If this hypothesis were correct, one should be able to observe the reappearance of the vacuolar, frothy type of interstitial cell in the hypertrophied gonads of chicks after exogenous gonadotrophic stimulation had been removed, and in the gonads of older birds when endogenous gonadotrophin secretion had been inhibited. The following experiments, using the single-comb, light-brown Leghorn fowl, were planned to test this hypothesis. A preliminary report of some of the results has been published (Taber, 1949b).

Gonads were fixed in a modified Maximow's fluid containing osmic acid, and were stained with anilin acid fuchsin and methyl green, and Mallory's triple connective tissue stain.

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EXPERIMENTS AND RESULTS

1. Effects of removal of exogenous gonadotrophic stimulation. Nine female and 14 male chicks were injected daily with 100 I.U. of pregnant mare serum (PMS), beginning the first day after hatching. Three females

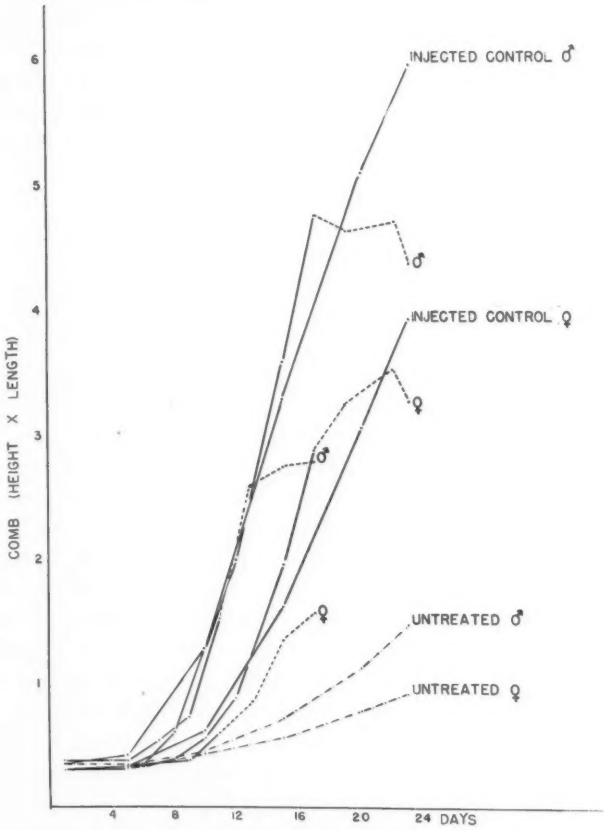

FIG. 1. Comb growth of untreated chicks (averages from 73 females and 70 males), controls injected daily with 100 I.U. of PMS for 23 days (averages from 24 females and 29 males), and of chicks similarly injected for 10 or 16 days, after which treatment was discontinued. Broken lines represent comb sizes following cessation of injections.

and one male received 10 injections and were killed on the 17th day, 6 days after the last injection, while 4 females and 4 males received 16 injections and were killed on the 23rd day, 7 days after the final injection. The remaining 2 females and 9 males were injected up to the day of autopsy and served as injected controls.

Figure 1 shows the average comb growth of untreated controls, controls injected with 100 I.U. of PMS daily for 23 days, and of the chicks killed 6 or 7 days after PMS injections were discontinued. Of the birds killed on the 17th day, the female combs showed a slight retardation in growth rate 6 days after injections were discontinued, while a more pronounced retardation was observed noted in the males by the 4th day. A similar retardation was within two days in the birds killed on the 23rd day.

Histological examination of the ovaries from 17-day-old untreated birds revealed discrete nests of interstitial cells, packed with osmic-stained globules, in the medullary region. These cells had the vacuolar, frothy appearance previously described (Taber, 1948a) (fig. 3). Ovaries from injected birds showed pronounced medullary hyperplasia, with distended medullary tubules, but no apparent changes in the cortex. In the injected controls, many groups of interstitial cells, containing small osmic and bluish-staining granules, were scattered through the medullary tissue and concentrated immediately below the cortex. No vacuolar cells or large osmicated droplets were observed (fig. 4). The gross histological appearance of the ovaries from the birds killed 6 days after injections were discontinued, was similar to that of the injected control, but the interstitial cells contained larger and more heavily osmicated droplets (fig. 5).

The ovaries of 23-day-old untreated birds resembled those of the younger controls, except that the interstitial cells contained fine osmic and basophilic granules rather than large droplets (fig. 6). The distention of the medullary tubules in the hyperplastic medulla of the injected control was such that the medullary tissue extended into the cortex and blister-like cysts appeared on the ovarian surface. Near such regions, the cortex was thin and no follicles were observed. No osmic and few bluish-staining granules were seen in the interstitial cells (fig. 7). For the most part their cytoplasm appeared clear, or seemed to contain large, clear vacuoles, separated by threads of lightly stained cytoplasm. The general appearance of the ovaries from the birds killed 7 days after treatment ceased was similar to that of the injected control, but the interstitial cells varied. These cells in the chick showing least retardation in comb growth resembled those in the injected control, while in the chick showing most pronounced comb regression, heavily osmic-stained, foamy cells characteristic of younger untreated controls were observed (fig. 8)

The interstitial cells in the testes of untreated 17-day-old males were grouped in clusters scattered between the tubules, and contained relatively large, osmic-stained droplets (fig. 9). In both the injected controls and the experimental group there was pronounced enlargement of the tubules as well as hyperplasia of the interstitial cells. These cells, in the

injected controls, were filled with fine osmic or basophilic granules (fig. 10), while in the experimental group large osmic-stained globules filled the cells (fig. 11).

The testes from 23-day-old untreated males contained interstitial cells

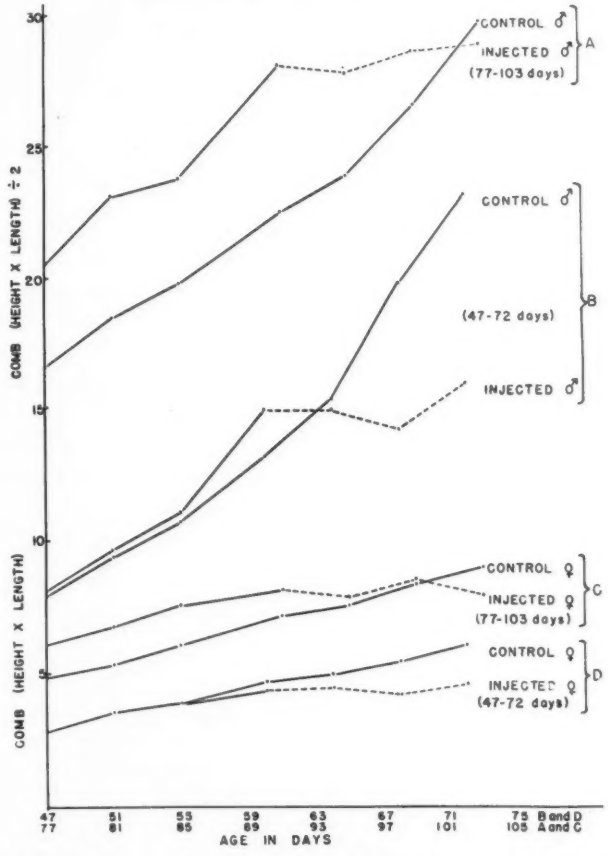

FIG. 2. Comb growth of control birds and of birds which received daily injections of 8 mg. of estradiol benzoate/100 gr. body weight. The injection periods are indicated by broken lines. Note that the days 47–75 on the ordinate refer to groups B and D, while the days 77–105 refer to groups A and C. Also, note that the comb figures plotted for group A represent the actual comb sizes divided by 2.

similar to those described for females of this age (fig. 12). As in the younger treated males, there were pronounced hypertrophy of the tubules and hyperplasia of the interstitial cells of the injected birds. The interstitial

cells of injected controls were granular and showed little osmication (fig. 13), while these cells in the experimental group were of the vacuolar type typical of recently hatched, untreated chicks (fig. 14).

2. Effects of estrogen injections. Four females and 6 males were given daily injections of 8 R.U. of estradiol benzoate per 100 gr. of body weight, beginning the 60th or 90th day, and continuing for 12 days. This amount of estrogen was sufficient to cause feminization of male plumage.

Retardation in the rate of comb growth was observed within 4 days after injections started (fig. 2).

Three injected females with 2 controls and 3 injected males with 2 controls were killed on the 72nd day, while one injected female with one control and 3 injected males with 3 controls were killed on the 103rd day. The oviducts of the younger treated females were larger than controls, but no change was detected in the oviduct of the older treated female.

No gross histological differences were observed between the ovaries of treated and control birds. In the 72-day-old controls, scattered nests of interstitial cells, containing fine osmic and basophilic granules, were observed in the ovarian stroma and in the connective tissue around the follicles (fig. 15). In the injected birds, these nests of cells appeared frothy or heavily laden with osmic-stained droplets (fig. 16). Both vacuolar and granular interstitial cells were observed in the 103-day-old control (fig. 17). The ovaries of injected birds showed an increase in the number of vacuolar cells and in the degree of osmication (fig. 18).

FIG. 3. Ovary×970 from 17-day-old untreated chick. Note vacuolar medullary interstitial cells.

FIG. 4. Ovary×970 from 17-day-old chick, injected 1 through 16th day with 100 I.U. PMS. Note granular cytoplasm of medullary interstitial cells.

FIG. 5. Ovary×970 from 17-day-old chick, injected 1 through 10th day with 100 I.U. PMS. Note osmic-stained droplets in medullary interstitial cells.

FIG. 6. Ovary×430 from 23-day-old untreated chick. Note medullary interstitial cells containing fine osmic-stained and basophilic granules.

FIG. 7. Ovary×430 from 23-day-old chick, injected 1 through 22nd day with 100 I.U. PMS. Note increased number of medullary interstitial cells, and lack of osmic-stained droplets.

FIG. 8. Ovary×430 from 23-day-old chick, injected 1 through 16th day with 100 I.U. PMS. Note increased number of medullary interstitial cells, all containing large osmic-stained droplets.

FIG. 9. Testis×430 from 17-day-old untreated chick. Note osmic-stained interstitial cells.

FIG. 10. Testis×430 from 17-day-old chick, injected 1 through 16th day with 100 I.U. PMS. Note lack of osmic-stained interstitial cells.

FIG. 11. Testis×430 from 17-day-old chick, injected 1 through 10th day with 100 I.U. PMS. Note osmic-stained interstitial cells.

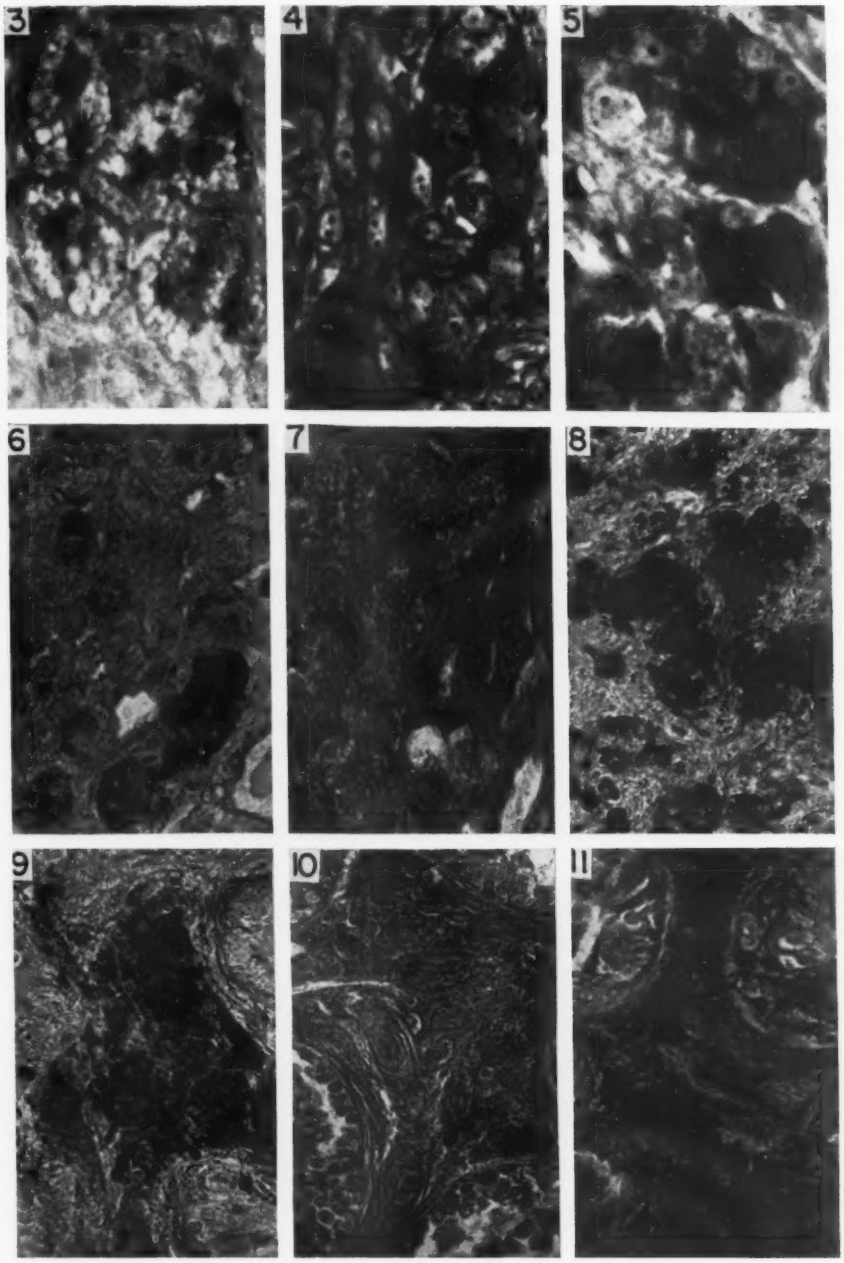

PLATE I

The seminiferous tubules of estrogen-injected males were smaller than those of untreated birds. The interstitial cells of the 72 and 103-day-old controls contained no osmic-stained granules or droplets (fig. 19), while in the injected birds of each age, the cells were filled with osmic-stained droplets and frequently appeared frothy (fig. 20).

Three females and 3 males were injected daily with 25 R.U. of estradiol benzoate for 24 days beginning on the 4th day after hatching, while 4 females and 2 males were given 100 R.U. daily, for 11 days, beginning on the second day.

The comb size of males receiving 25 R.U. averaged 2.92 (height ×length, in cm.) on the 28th day as compared with 3.71 for controls. The combs of estrogen-treated females (3.62), however, were larger than those of controls (1.67). In the birds receiving 100 R.U. of estradiol benzoate, the combs of females (.61) and males (.82) were smaller than those of untreated female and male chicks, measuring .80 and 1.26, respectively.

In both groups the ovaries and testes of controls contained interstitial cells filled with fine osmic-stained and basophilic granules. The number of cells containing osmicated droplets, as well as the size of these droplets was increased in estrogen-treated chicks. Occasionally, groups of cells containing bright red granules distributed throughout the cell were noted in the medulla of treated ovaries. Such cells were rarely observed in control ovaries or in the testes of injected or untreated chicks.

3. Effects of androgen injections. Five females and 6 males were given daily injections of .75 mg. of testosterone propionate for 24 days beginning

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FIG. 12. Testis×970 from 23-day-old untreated chick. Note osmic-stained interstitial cells.

FIG. 13. Testis×970 from 23-day-old chick, injected 1 through 22nd day with 100 I.U. PMS. Note lack of osmic-stained droplets in interstitial cells.

FIG. 14. Testis×970 from 23-day-old chick, injected 1 through 16th day with 100 I.U. PMS. Note vacuolar interstitial cells.

FIG. 15. Ovary×970 from 72-day-old untreated bird. Note granular medullary interstitial cells.

FIG. 16. Ovary×970 from 72-day-old bird, injected for 10 days with estradiol benzoate. Note vacuolar interstitial cells.

FIG. 17. Ovary×430 from 103-day-old untreated bird. Note occasional presence of fine osmic-stained granules in medullary interstitial cells.

FIG. 18. Ovary×430 from 103-day-old chick injected for 10 days with estradiol benzoate. Note groups of osmic-blackened medullary interstitial cells.

FIG. 19. Testis×430 from 72-day-old untreated bird. Note lack of osmic-stained droplets in the relatively few interstitial cells.

FIG. 20. Testis×430 from 72-day-old bird, injected for 10 days with estradiol benzoate. Note osmic-blackened interstitial cells.

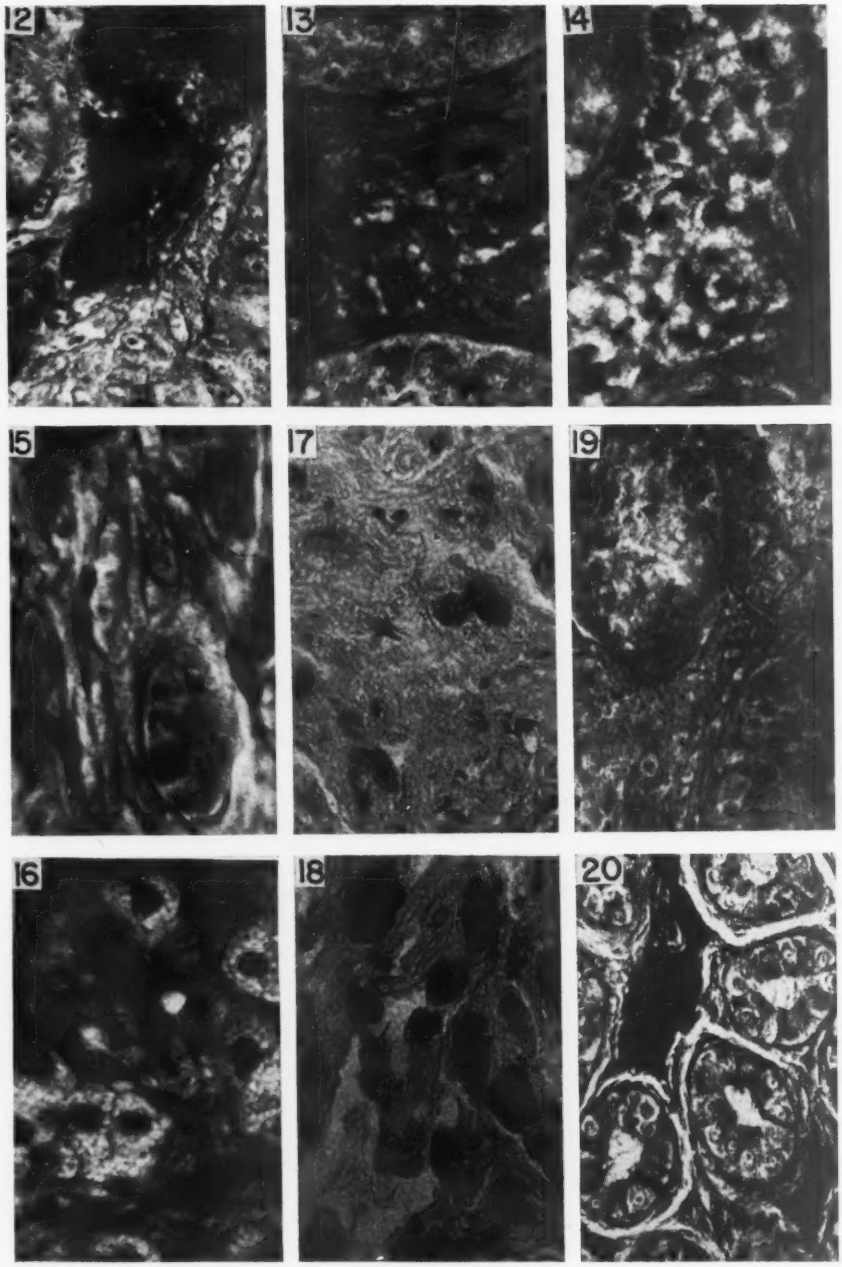

PLATE II

on the 4th day after hatching. There appeared no significant difference in the degree of comb stimulation in males and females, and crowing was induced in both sexes after two injections.

In the ovaries of these treated birds there appeared no increase in the number of osmic-stained cells, but the osmic droplets were significantly larger than those in untreated ovaries, and many groups of cells containing brilliantly stained red granules were observed scattered through the medullary region.

The testes of androgen-treated birds could not be distinguished from those of estrogen-treated males.

DISCUSSION

Since the medullary component of the ovary is homologous to the testis, and since the normal ovary produces sufficient androgen to cause comb growth in the fowl, one might suspect that similar glandular cells could be identified in the ovarian medulla and in the testis, and that the presence of these cells could be correlated with androgen secretion. Such appeared to be the case in ovaries and testes of untreated birds of different ages and of birds in which androgen production was precociously induced by gonadotrophin treatment (Taber, 1948a, 1948b, 1949a).

Gonadotrophic hormone stimulation resulted in a hyperplasia of interstitial cells in the testis and in medulla of the ovary, as well as a change in their appearance from the vacuolar lipoid-filled type characteristic of gonads which were secreting relatively small amounts of androgen to a cell containing basophilic granules and relatively few or no osmic-staining droplets. If gonadotrophin injections were discontinued 6–7 days before autopsy, an increased number of interstitial cells, compared with gonads from untreated birds, could be observed. However, since these interstitial cells were filled with large osmic-blackened droplets similar to those observed in the inactive gonads of young birds, and since a reduction in the rate of comb growth was observed, one can suspect that these cells were of an inactive type.

Injections of estrogen resulted in a decreased comb growth rate. This was not due to a direct inhibitory action of estrogen on the comb, but presumably to an indirect action through the pituitary. With the release of pituitary gonadotrophins inhibited by the high level of estrogen, the ovaries and testes were not stimulated to produce androgen, and consequently, comb regression followed. The interstitial cells in gonads of birds treated in this way contained the lipoid-filled globules typical of the inactive secretory phase.

These experiments have shown a correlation between the secretion of an-

drogen, as measured by comb growth, and the presence of interstitial cells containing basophilic granules. Lack of androgen secretion was correlated with the presence of osmic-blackened interstitial cells.

That the presenc of androgen was not the stimulus for the appearance of the basophilic type of interstitial cell was demonstrated by experiments in which the amount of androgen was increased by injections of testosterone propionate. Increasing the androgen did not result in an increment of basophilic cells, but rather in an increase in the size and number of lipoid droplets in the cells. It would seem that androgen acted similarly to estrogen, inhibiting the gonads by repressing the pituitary. The significance of the increased number of medullary interstitial cells containing prominent red-staining granules in the ovaries of androgen-treated birds is not clear.

The results of these experiments support the hypothesis that the interstitial cells in the fowl testis and in the medulla of the fowl ovary are a major source of androgen.

SUMMARY AND CONCLUSIONS

The withdrawal of exogenous gonadotrophic stimulation from the single-comb, light-brown Leghorn fowl resulted in regression of the comb, indicating decreased androgen production, and in the reappearance of foamy, lipoid-filled interstitial cells in the testes and in the medulla of the ovaries.

Inhibition of secretion of endogenous gonadotrophins by estrogen injections produced similar results.

That lack of androgen was not a causal factor for the appearance of these lipoidal cells was shown when injections of androgen were followed by effects essentially the same as those observed following estrogen treatment.

The results of these experiments provide additional evidence that the interstitial cells of the testis and similar cells in the medullary component of the ovary are responsible for androgen production in the single-comb, light-brown Leghorn fowl; that interstitial cells filled with basophilic granules represent an active secretory phase, while those containing large osmic-staining, lipoidal droplets represent an inactive phase.

Acknowledgments

Grateful acknowledgment is made to Dr. M. H. Kuizenga of the Upjohn Company for the pregnant mare serum powder, and to Dr. Norman L. Heminway of the Schering Corporation for the estradiol benzoate (Progynon-B) and the testosterone propionate (Oreton) used in these experiments.

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MAINTENANCE OF PREGNANCY IN INTACT RABBITS IN THE ABSENCE OF CORPORA LUTEA[1]

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IT IS well known that the secretion of the corpus luteum, progesterone, plays an important part in the proliferation of the uterine endometrium, the growth and implantation of the ovum, the formation of the placenta, and the maintenance of pregnancy. In the rabbit, removal of the corpora lutea interrupts pregnancy while the administration of luteal extract (or progesterone) maintains gestation right up to the full term in the ovariectomized animals (Allen and Corner, 1929). Whether or not pregnancy can be maintained in an intact, non-ovulated animal by the administration of progesterone has not previously been determined. The possibility of such maintenance can be only investigated by transferring fertilized ova into a non-ovulated animal.

In a previous investigation on the development of transferred rabbit ova in relation to the ovulation time of the recipient rabbits (Chang, 1950b), it was observed that ova at different stages (1 to 6 days after mating) could develop into normal young only when transferred to the recipients whose luteal stage more or less corresponded to the stage of the transferred ova. For successful ova transfer, therefore, the proper luteal stage of the recipient must be determined. Since the majority of mammals ovulate spontaneously, synchronization of ova age and luteal age cannot be as easily adjusted, as with rabbits. In order to practice ova transfer in animal breeding, it is therefore of importance to determine the possibility of pregnancy in the intact, non-ovulated animal by the administration of progesterone.

ANIMALS AND PROCEDURES

The animals used were adult rabbits of mixed breeds bought from local dealers. The ova donors were superovulated and inseminated in order to obtain a large number of fertilized ova for transplantation. The recipients were of the same stock, but had been

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kept in the laboratory for a longer time. The procedure of superovulation (Pincus, 1940; Chang, 1948), the technique of insemination (Macirone and Walton, 1938; Walton, 1930), the methods of recovery of ova at early or at late stages and the methods of transfer have been described elsewhere (Chang, 1948, 1950a).

In the present investigation, in most cases, 1-day-old ova from albino rabbits were transferred to the left tube of a recipient. Five days later, the 6-day-old blastocysts (6-day ova) from colored rabbits were transferred to the right uterus. At operation, the ovaries of the recipients were examined to ascertain the absence of corpora lutea. Half of the recipients were sacrificed or laparotomized 12 to 22 days after the transfer of 1-day ova or 7 to 17 days after the transfer of 6-day ova. The normal embryos were counted and the uterine swellings of various sizes which indicate the degeneration of the embryos at various stages, were measured and examined. About half of the animals were allowed to deliver at full term.

In order to determine the proper dosage of progesterone for the maintenance of pregnancy, and the appropriate method of administration, four series of experiments were performed: (1) progesterone[2] in sesame oil was injected subcutaneously daily in increasing dosage (1 mg. daily for 6 to 8 days, 1.5 mg. for 6 to 8 days, 2 mg. for 8 to 15 days). (2) Macrocrysta'line progesterone[3] was injected subcutaneously three times in increasing dosage (6.25 mg., 12.5 mg., 18.75 mg.) at intervals of one week. (3) Twenty-eight to 35 mg. of progesterone in Ca gluconate[4] were implanted under the skin. (4) Twenty-five mg. of macrocrystalline progesterone[3] were injected subcutaneously one to three times at intervals of 8 to 10 days.

The first daily injection of progesterone was given one day before the transfer of the 1-day ova or 5 to 6 days before the transfer of the 6-day ova. The implantation of progesterone tablets and the injection of macrocrystalline progesterone were performed in most cases at the time of transfer of the 1-day ova or 5 days before the transfer of the 6-day ova.

As the study progressed, it was realized that most of the pregnant recipients could not deliver the litter properly, i.e., parturition lasted for 3 to 15 days in some animals. Thus a few animals were injected subcutaneously with 1 mg. of stilbestrol followed by an intravenous injection of .5 cc. of posterior pituitary extract.[5]

RESULTS

Figure 1 shows the ovaries (without corpus luteum but with median sized follicles) and fetuses in the uteri of a recipient. It is clear that by administration of progesterone, the normal development of transferred ova can be maintained in the intact rabbit in the absence of corpora lutea. The results of this study are summarized in Table 1. The daily injection of a small dose (1–2 mg.) is very ineffective as is shown by the low percentage of normal development (2.4–4.2) obtained. A relatively high dose (6–19 mg.), administered three times during gestation, increased slightly

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[2] Lutocylin supplied by Ciba Pharmaceutical Products.

[3] Macrocrystalline suspension of progesterone supplied by Ciba Pharmaceutical Products.

[4] Prepared by Dr. G. Pincus.

[5] Pitocin manufactured by Parke Davis Company.

the percentages of normal development (9 to 14). The implantation of a high dose of progesterone (28–35 mg.) increased the percentage of normal development to a certain extent (10–28), while the injection of one high dose (25 mg.) of macrocrystalline progesterone further improved the percentage of normal development (17.5–36%). However, the injection of 25

FIG. 1. Reproductive tracts of a recipient rabbit. Six 1-day ova were transferred to the left tube. Five days later, eight 6-day ova were transferred to the right uterus. 25 mg. of macrocrystalline progesterone were injected subcutaneously twice: the first injection at the time of transfer of the 1-day ova; the second injection, 9 days later. The recipient was sacrificed 16 days after the transfer of the 1-day ova. Four of the six 1-day ova developed into normal embryos; two of them failed to implant. Six of the eight 6-day ova implanted, but 2 of them degenerated (near Fallopian tube). Note the absence of corpora lutea and the presence of medium sized follicles on the ovaries.

mg. two or three times during pregnancy gave the best results (37–50%). The relatively low percentage of development (10–28%) in the case of implantation of a large quantity in tablet form is perhaps due to the slow rate of absorption. It is interesting to note that the percentage of transferred ova developing normally seems far more dependent on the administration of an initial high dose than on the total amount given.

TABLE 1. IMPLANTATIONS AND NORMAL DEVELOPMENT OF TRANSFERRED OVA IN NON-OVULATED RABBITS BY ADMINISTRATION OF PROGESTERONE

Ova	Total no. recip.	Total no. ova	Treatment		Sacrificed or laparotomized			Allowed to deliver			Percentages		
			Method admin.	Total dose, mg.	No. recip.	Degen. embryo	Normal embryo	No. recip.	Young dead before birth	Normal young	Total implant.	Degen. or dead at var. stages	Normal develop.
1 day old	5	71	daily inj.	31–50	4	1	2	1		1	5.6	1.4	4.2
	4	29	3 inj.	37.5	2	10	0	2		4	48.3	34.5	13.8
	4	32	1 imp.	28–35	4	6	9	1[1]		1	46.8	18.7	28.1
	6	47	1 inj.	25	3	4	12	3		5	44.7	8.5	36.2
	6	48	2 inj.	50	1	0	4	5	7	14	52.1	14.6	37.5
	6	46	3 inj.	75	3	3	11	3	5	12	67.4	17.4	50
6 day old	4	42	daily inj.	31–50	4	21	1	1[2]		1	52.4	50.0	2.4
	4	23	3 inj.	37	2	9	0	2	3	2	60.9	52.2	8.7
	4	20	1 imp.	28–35	4	4	2				30.0	20.0	10.0
	6	40	1 inj.	25	3	7	6	3		1	35.0	17.5	17.5
	5	43	2 inj.	50	4	9	16	2[3]	1	4	60.5	23.2	37.2
	6	26	3 inj.	75	3	4	6	3	1	5	61.5	19.2	42.3

[1] One normal and 1 degenerated embryo observed at laparotomy.
[2] One normal and 7 degenerated embryos observed at laparotomy.
[3] Four normal and 3 degenerated embryos observed in the laparotomized animal.

The percentages of implantation and of degeneration of ova (or death of the embryo) at various stages after implantation, are also presented in Table 1. When daily small doses were administered, the percentage of implantation was very low (5.6) in the case of 1-day ova, as compared with 6-day ova (52%). Since the 1-day ova were transferred to the tubes, while the 6-day ova were transferred to the uterus, the failure of implantation in the case of the 1-day ova can be attributed to a failure of reception and transportation from tube to uterus. As the initial dosage increases the percentage of implantation increases in the case of 1-day ova. This demonstrates that the proper reception and transportation of ova in the tube depends upon the stimulation provided by a large dose of progesterone. Beside this point, there is no striking difference in percentage of implantation between 1-day ova and 6-day ova. The percentage of implantation, however, was higher in both cases when a heavy dose of progesterone was administered twice or three times.

In calculating the percentage of ova which degenerated after implantation, all premature fetuses or those which were dead at various stages before birth, and uterine swellings of various sizes (10–14 mm.) are included. The percentage of degeneration is high (50%) in the case of transferred 6-day ova when a daily low dose was administered. In this case only small implantation sites were observed. This indicates the early degeneration of the implanted ova. Degeneration is also high (35–52%) when a relatively low dose (6–19 mg.) was administered 3 times as compared with a high dose (25 mg.) administered 1 to 3 times (9–23%) during pregnancy. This suggests once again that the maintenance of normal development requires an initial high dose of progesterone while subsequent treatment prevents the degeneration of the embryos or death of the fetus.

Thirteen animals were allowed to deliver naturally. Three of them made a nest at the time of parturition. The length of gestation was very irregular, varying from 25 to 44 days after the transfer of 1-day ova or 20–30 days after the transfer of 6-day ova. Five of these 13 animals had been injected with 25 mg. of macrocrystalline progesterone on two occasions; the parturition of four of them lasted from 3 to 15 days. None of these 13 animals nursed their young though milk could be squeezed from their teats. Four animals were injected with posterior pituitary extract in combination with stilbestrol at the 31st day after the transfer of 1-day ova. They delivered about 10 minutes after injection. Two of them made nests but only one nursed the young up to the time of weaning.

DISCUSSION

In a study of the development and fate of transferred ova of different ages in relation to the luteal stage of the recipients (Chang, 1950b), it

was reported that there is little or no chance for a transferred ovum to develop into young if placed in the tract one or two days before or after the corresponding luteal stage. Since the physiological condition of the female tract at pregnancy is controlled mainly by progesterone, the low percentage of development observed in some groups in the present study was probably due to inadequate treatment and thus the physiological condition of the tracts was not adequate for the development of the transferred ova.

The low percentage of implantation of transferred 1-day ova observed after daily administration of a low dose of progesterone is similar to the condition which arises when 1-day ova are transferred to a rabbit at oestrus (Chang, 1950b). This indicates that the administration of 1 mg. daily for 1 or 2 days is not sufficient to render the Fallopian tube of a non-ovulated rabbit as receptive as those of rabbits in the first day of pseudopregnancy.

The small implantation sites observed, and the low percentage of development of transferred 6-day ova under daily administration of low doses of progesterone is similar to the condition seen when 6-day ova are transferred to the uterus of a recipient in the third or fourth day of pseudopregnancy (Chang, 1950b). This indicates a low dose treatment, continued for 5 of 6 days, only renders the endometrium similar to that of a rabbit in the 3rd or 4th day of pseudopregnancy.

The percentages of normal development of 1-day and 6-day ova transferred to the recipient at the 1st and 6th day of pseudopregnancy is 54 and 42 (Chang, 1950a). The figures for transferred 1-day ova and 6-day ova were 37 to 50 and 37 to 42 when 25 mg. of macrocrystalline progesterone were administered twice or three times to the non-ovulated recipients. This shows that pregnancy may be maintained with the same efficiency by progesterone in ova transfer experiments as by using pseudopregnant animals.

In the ovariectomized rabbit, it is known that 0.2 mg. of progesterone daily is required to produce partial progestational proliferation of the endometrium in 5 days (Corner, 1937). According to early figures, 0.5 to 2 mg. is required for the maintenance of pregnancy (Corner and Allen, 1930; Pincus and Werthessen, 1938). Later investigators, however, have determined that while in the early stages about 1 mg. per day is sufficient, in later stages up to 5 mg. of progesterone daily is necessary (Allen and Heckel, 1939; Courrier and Kehl, 1938). Thus the total dose of progesterone required for the maintenance of pregnancy in the ovariectomized rabbit is about 120 mg. In the intact rabbit, as revealed in the present study, a total dose of 50 to 75 mg. is sufficient. Since it is not the total dose, but the initial high dose that contributes most to the maintenance of pregnancy in the intact rabbit, the low total dose required does not imply that secre-

tion of ovarian tissue other than corpus luteum plays a part in the maintenance of pregnancy.

According to Corner (1937), the model daily output of progesterone of a pregnant rabbit is 1.6 mg. Thus the total output of progesterone during pregnancy would be $1.6 \times 32 = 51.2$ mg., which may be the normal requirement for maintenance during the complete period of gestation. It is interesting to note that in the present study, a total dose of 50 mg. was sufficient to maintain pregnancy in an intact non-ovulated rabbit. This dosage may be close to the optimal requirement for the maintenance of pregnancy.

In the ovariectomized rabbit, for maintenance of pregnancy, a low daily dose (1 mg.) is sufficient in the early stages, but a high dose (5 mg.) is necessary in the late stages. In the intact rabbit, however, a heavy dose is required in the early stages. It is known that progesterone acts synergistically with estrogen (Allen, 1937; Pincus and Werthessen, 1938) and that a definite ratio between progesterone and estrogen (750:1) is required for the maintenance of pregnancy in the ovariectomized rabbit (Courrier and Kehl, 1938). In order to build up the physiological condition of the Fallopian tubes and uteri for pregnancy in an intact, non-ovulated rabbit, the initial large dose of progesterone is probably required to compensate for the large amount of estrogen produced in the ovaries.

Although the absorption (Foss, 1942) and the metabolism (Zondek, 1939) of progesterone is rapid, the prolongation of gestation and the difficulty in parturition observed in this study demonstrate the prolonged effect of macrocrystalline progesterone, i.e. more than 20 days in the group of animals that received two injections.

It would be speculative to explain why only a few animals made a nest and only one animal nursed her young though most of them secreted milk. Histological sections of three ovaries of recipients sacrificed at late pregnancy show no obvious difference as compared with those of untreated animals.

SUMMARY

One-day and 6-day old rabbit ova were transferred respectively to the tube and uterus of non-ovulated, intact rabbits treated with progesterone, in order to determine the probability of maintenance of pregnancy in the absence of corpora lutea. Thirty-six to 50% of the transferred 1-day ova or 18 to 42% of the transferred 6-day ova developed into normal young when 25 mg. of macrocrystalline progesterone were injected subcutaneously one to three times during pregnancy. When a daily small dose was administered (1–2 mg.), a low percentage of implantation (5.6) and development (4.2) were observed in the transferred 1-day ova; but a high percentage of implantation (52) and a low percentage of development (2.4)

were observed in the transferred 6-day ova. When a medium dose was injected (6–19 mg.) three times during pregnancy or a high dose (28–35 mg.) was implanted once, the percentage of ova implantation was high (30–60), while that of development was rather low (9–28%) in the case of both 1-day ova and 6-day ova. It seems that the initial large dose is important for the reception and transportation of ova from the tube to the uterus and the maintenance of pregnancy after implantation. The length of gestation was very irregular and parturition lasted for several days. Only a few recipients (5 of 17) made nests and only one nursed her young although milk was produced in most of the animals.

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ESTROGEN AND ALKALINE PHOSPHATASE ACTIVITY IN THE GENITAL TRACT OF THE MALE MOUSE[1]

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RECENTLY considerable attention has been focussed on the hormone-enzyme relationship by several groups of investigators using histochemical and cytochemical methods (Kochakian, 1950). The hormonal control of phosphatase activity in several areas of the male genital tract has been studied by the Gutmans (1938, 1939), Pazos and Huggins (1945), Huggins (1947), Atkinson (1948), Soulairac and Thibault (1948a, b), Dempsey (1948), Dempsey *et al.* (1949), Wislocki (1949), Stafford *et al.* (1949), and Nava and Fiore (1949). According to certain of these workers, castration of male mammals results in at least partial loss of phosphatase activity from the genital tract which can be restored by androgen replacement therapy. The present study was undertaken to determine the effects of estrogen on alkaline phosphatase activity in genital tissues of the male mouse, using a cytochemical technic.

MATERIALS AND METHODS

Seventy-nine male mice were used during the course of this investigation. They were of several strains and ranged in age from 8 to 20 weeks at the time experiments were begun. Consistent differences ascribable to age and strain were not noted, although individual variations within each age group occurred. The total number was divided into 4 major groups as follows: 16 intact controls; 27 untreated castrates; 25 castrates treated with estrogen; 11 intact mice treated with estrogen.

Bilateral castration was performed through the scrotum and estrogen treatment was started immediately by subcutaneous implantation of α-estradiol pellets weighing one to three mg. Duration of treatment before sacrifice of individual members of the several experimental groups is indicated in Table 1.

Various areas of the genital tract were removed and fixed in chilled absolute acetone. Paraffin sections were cut at 6μ, and alkaline phosphatase activity was demonstrated by incubation in glycerophosphate for 18 hours at pH 9.5 (approximately) according to Barger's modification of the Gomori technic as employed by Bern (1949b). No attempt was made to demonstrate acid phosphatase cytochemically due to the unreliability of the technic (Lison, 1948). Only cytoplasmic evidences of alkaline phosphatase activity were recorded, inasmuch as nuclear activity had been found little in evidence in previous studies (Bern, 1949b). In addition, Ruyter and Neumann (1949) question the reality of nuclear alkaline phosphatase as routinely demonstrated. Particular attention was paid to changes occurring in the vas deferens, seminal vesicle, anterior prostate (coagulating gland), and dorsal prostate.

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OBSERVATIONS

Table 2 summarizes very briefly the salient observations concerning the hormonal control of alkaline phosphatase activity in the genital tract of male mice.

Untreated intact mice. The phosphatase activity apparent in the normal untreated mouse seminal vesicle, anterior and dorsal prostates, and vas deferens is "stromal" (Bern, 1949b); that is, it is confined to connective

TABLE 1. DURATION OF TREATMENT OF INDIVIDUAL MICE

Group	Total No.	No. of mice per period of treatment and/or castration in weeks							
		1	2	3	4–5	6	7	8–9	13
Untreated castrates	27	1	2	5	5	8	3	2	1
Castrates treated with estrogen	25	4	4	6	3	6		2	
Intact mice treated with estrogen	11			3	1	2	3	2	

TABLE 2. ALKALINE PHOSPHATE DISTRIBUTION IN THE GENITAL TRACT OF MALE MICE IN DIFFERENT ENDOCRINE STATES

Animal group	Seminal vesicle		Vas deferens		Anterior and dorsal prostates		
	Fibro-muscular wall	Sub-epithelial stroma	Fibro-muscular wall	Sub-epithelial stroma	Stroma	Meta-plastic epithelium	Original epithelium
Normal Intact	+	+	+	+	+		−
Castrate	−	+	−	+	+ or −		−
Castrate +Estrogen	+	+	+	+	+ or −	+	−
Intact +Estrogen	+	+	+	+	+	+	−

tissue and muscular elements (and associated small blood vessels). It is almost completely absent from the epithelium (figures 1 and 4).

Castrated mice. Castration results in the virtual disappearance of phosphatase activity from certain regions along with its definite retention in other regions of the organs studied. In the seminal vesicle of castrates, activity is generally restricted to the subepithelial stroma and the innermost muscularis and is lost in the majority of the fibromuscular wall. In the vas deferens, the fibromuscular wall becomes devoid of activity, but the subepithelial stroma retains intense activity (figure 2). Although stromal elements atrophy in the prostatic lobes, phosphatase activity generally remains in some of the remaining circumalveolar fibromuscular tissue.

Estrogen-treated castrated mice. Estrogen administration to castrates results in the retention of considerable stromal phosphatase activity. The fibromuscular walls of the seminal vesicle and of the vas deferens (figure 3) are generally intensely active throughout their extent, very much as in the normal animal. The fibromuscular hypertrophy usual in male sex accessories as a result of estrogen administration is evident. Stromal phospha-

tase is also often noticeable in the prostatic lobes, but occasionally is almost totally absent (figures 5 and 6).

The epithelial changes described first and in detail by Lacassagne (1933) and by Burrows (1935) occurred as a result of estrogen treatment. Squamous metaplasia was particularly evident in the anterior prostate, many of the alveoli of which transformed into masses of keratin surrounded by pluristratified squamous epithelium (figure 9). The beginnings of such metaplastic changes (principally pluristratification and basal cell proliferation) were noted also in the dorsal prostate (figures 5 and 6), in the duct of the seminal vesicle, and occasionally in the body of the latter (figure 10).

The lack of alkaline phosphatase activity in the epithelium of the seminal vesicle and dorsal and anterior prostates of intact and castrated mice is reversed by estrogen treatment. The metaplastic epithelium shows intense activity, and the greatly transformed alveoli of the anterior prostate are sometimes entirely alkaline phosphatase positive (figure 9). Generally, activity is confined to the more basal cell layers in extensively cornified alveoli. In prostatic alveoli and in areas of the seminal vesicle wherein the metaplastic process is not fully completed, the intensely positive replacing epithelium stands out in contrast to the negative original epithelium (figures 5, 6, 8 and 10). It thus becomes possible to distinguish between the mitotically active basal cells and the original epithelium by what almost amounts to a differential stain, based on alkaline phosphatase activity.

Estrogen-treated intact mice. No consistent differences between intact and castrated mice receiving estrogen were noted. The metaplastic process in general was as extensive in the intact animals as in the castrates and was characterized by intense alkaline phosphatase activity (figure 7). Similarly, stromal activity was retained in the vas and seminal vesicle as in the untreated intact animal.

DISCUSSION

Some recent studies have attempted to correlate alkaline phosphatase activity with synthesis of fibrous proteins. Danielli (1946) has suggested this in regard to tissue regeneration, and Bradfield (1946, 1949) has discussed extensively the possible relationships between alkaline phosphatase, nucleic acids and fibrous protein synthesis, basing his discussion upon studies of protein secretion in invertebrates. Jeener (1947, 1948) suggested that increased alkaline phosphatase activity may be associated with the synthesis of keratin, collagen and myosin.

In the mouse, the absence of alkaline phosphatase from the superficial cell layers of many coagulating gland alveoli wherein keratinization has been most extensive and its virtual absence from the epidermis (Biesele

and Biesele, 1944, and personal observations) suggest that the enzyme may not be present in association with keratin *per se*, but rather with keratin that is being actively synthesized. Johnson *et al.* (1945) noted disappearance of alkaline phosphatase activity in rat hair follicles after differentiation had been completed, and Jeener (1948) observed a decrease in activity of the superficial epithelial layers and of the keratin in the mouse vagina when estrogen treatment was continued for three days.

The retention of alkaline phosphatase activity in the subepithelial stroma of the seminal vesicle in castrate mice noted above disagrees with earlier observations by Atkinson (1948), who reported total disappearance 10 days after castration. Soulairac and Thibault (1948a, b) described disappearance of all genital tract alkaline phosphatase after castration in the male rat. Dempsey (1948) and Dempsey *et al.* (1949) demonstrated disappearance of glycerophosphatase activity in the rat seminal vesicle one month after hypophysectomy or gonadectomy, although fructose diphosphatase activity persisted for a longer time (up to 4 months). Differences in the duration of incubation of the tissues, as well as species differences, may account for the disagreement. The ability of estradiol to restore alkaline phosphatase activity partially in castrate rats was noted by Soulairac and Thibault (1948b). In the mouse we have found that estrogen maintains fibromuscular alkaline phosphatase localizations in the seminal vesicle and vas deferens. Inasmuch as fibromuscular hypertrophy in these organs occurs as a result of estrogen treatment, Jeener's (1948) suggestion

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Description of Figures

(All photomicrographs are of paraffin sections of acetone-fixed tissues, 6μ thick, with nuclear coloration largely due to hematoxylin and dense extranuclear coloration to alkaline phosphatase activity.)

Fig. 1. Vas deferens of normal untreated intact mouse. Note phosphatase activity throughout fibromuscular wall including the subepithelial stroma and its absence from the epithelium. ×55.

Fig. 2. Vas deferens of mouse castrated for 38 days. Note disappearance of phosphatase activity from fibromuscular wall, retention in subepithelial stroma. ×55.

Fig. 3. Vas deferens of mouse castrated and treated with estradiol for 38 days. Note intense activity of hypertrophied fibromuscular wall. ×55.

Fig. 4. Seminal vesicle (at left) and anterior prostate (at right) of normal mouse. Note stromal phosphatase, absence of epithelial activity. ×55.

Fig. 5. Dorsal prostate alveoli of mouse castrated and treated with estradiol for 39 days. Note phosphatase-active basal cells, negative original epithelium, virtually inactive stroma. ×350.

Fig. 6. Dorsal prostate alveolus of mouse castrated and treated with estradiol for 21 days. Note phosphatase-active basal cells and circumalveolar stroma, negative original epithelium. ×350.

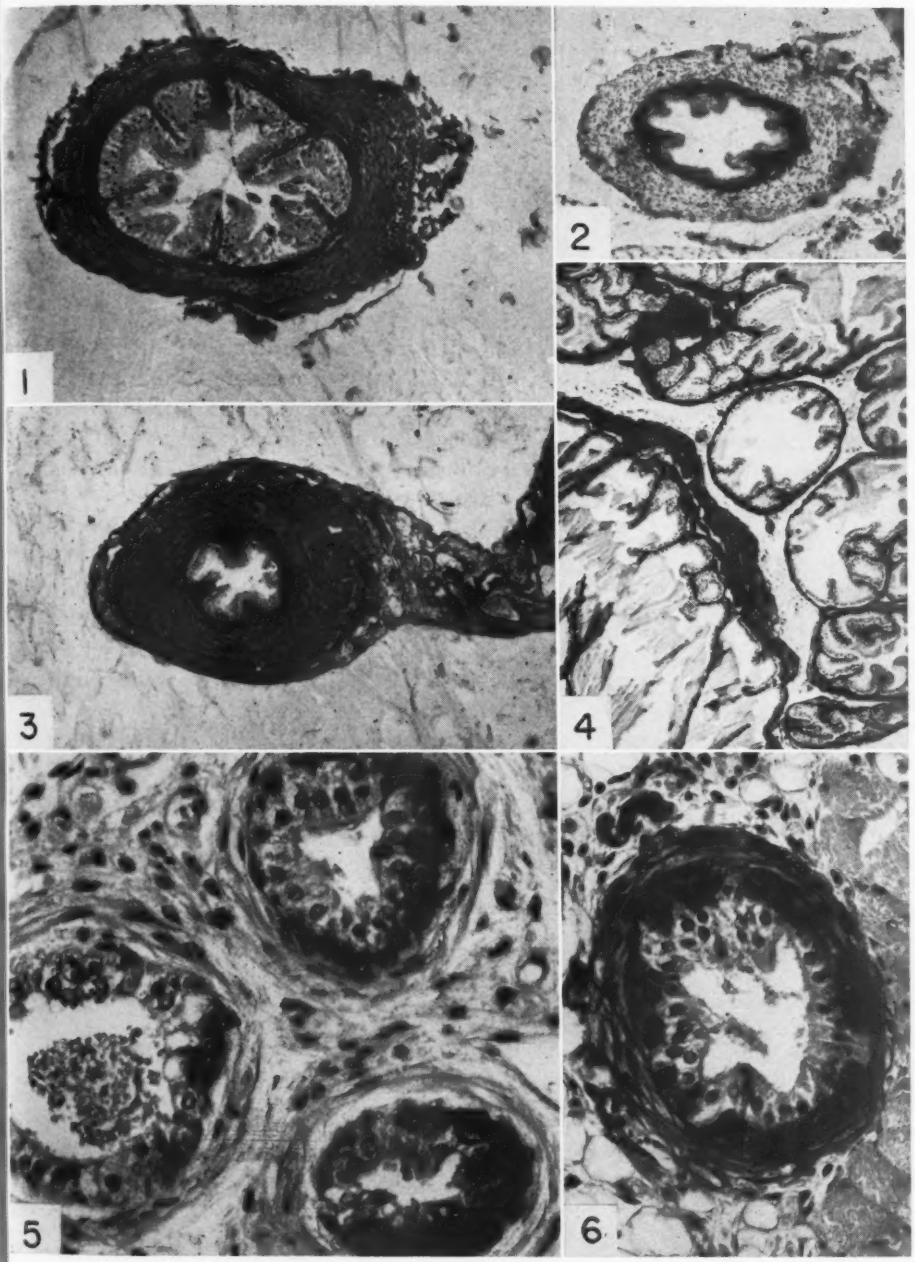

PLATE I

that alkaline phosphatase activity is associated with myosin synthesis provides a possible explanation. In these special areas one might consider that, in terms of a specific physiologic activity, estrogen shows a limited ability to replace androgen, a concept for which there is appreciable morphologic support (see Bern, 1949a).

Controversy exists in the literature as to the number of distinct mammalian phosphatases with optimal activity around pH 9.5 (*e.g.*, Gomori, 1949, 1950; Newman *et al.*, 1950). The data presented here suggest that genital alkaline phosphatase activities may be differentiated in part on the basis of reactions to sex steroids. Thus, activity in the subepithelial stroma of the seminal vesicle and vas deferens seems to be relatively independent of androgen, whereas that of the fibromuscular wall disappears in the absence of testicular androgen or a substituting estrogen. A similar situation may obtain in the female mouse uterus where castration results in the disappearance of alkaline phosphatase from the circular muscle but not from the longitudinal muscle or the subepithelial stroma (Atkinson and Elftman, 1946, 1947; Jeener, 1948).

Fuenzalida (1949a, b) demonstrated that rapidly proliferating, atypical (metaplastic?) epithelial growths in the guinea pig uterus are intensely alkaline phosphatase-active. Our observations on the male mouse indicate that intense phosphatase activity of the replacing epithelium in glandular areas undergoing metaplasia in response to estrogen can serve to clearly delineate these actively dividing cells from the original inactive epithelium. The basal cells in their first appearance are closely associated with and often almost indistinguishable from stromal elements. Their continued multiplication beneath the old epithelium results in the characteristic appearance of clusters of basal cells, as illustrated in figures 5 to 8. In view of Andrew and Andrew's (1949) recent contentions concerning the transformation of lymphocytes into ordinary epithelial cells in the epidermis,

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DESCRIPTION OF FIGURES

FIG. 7. Anterior prostate alveoli of intact mouse treated with estradiol for 21 days. Note islands of phosphatase-active basal cells, moderately active stroma. ×350.

FIG. 8. Anterior prostate alveolus of mouse castrated and treated with estradiol for 15 days. Note extensive metaplasia and intense phosphatase activity of replacing cells (some diffusion is evident); active stroma. ×350.

FIG. 9. Anterior prostate alveolus of mouse castrated and treated with estradiol for 64 days. Note complete transformation into keratinized mass, limited activity of most of the superficial cell layers, inactive stroma. ×350.

FIG. 10. Area of seminal vesicle undergoing metaplasia from mouse castrated and treated with estradiol for 38 days. Note intensely active replacing epithelium in contrast to inactive sloughing original epithelium; active subepithelial stroma. ×350.

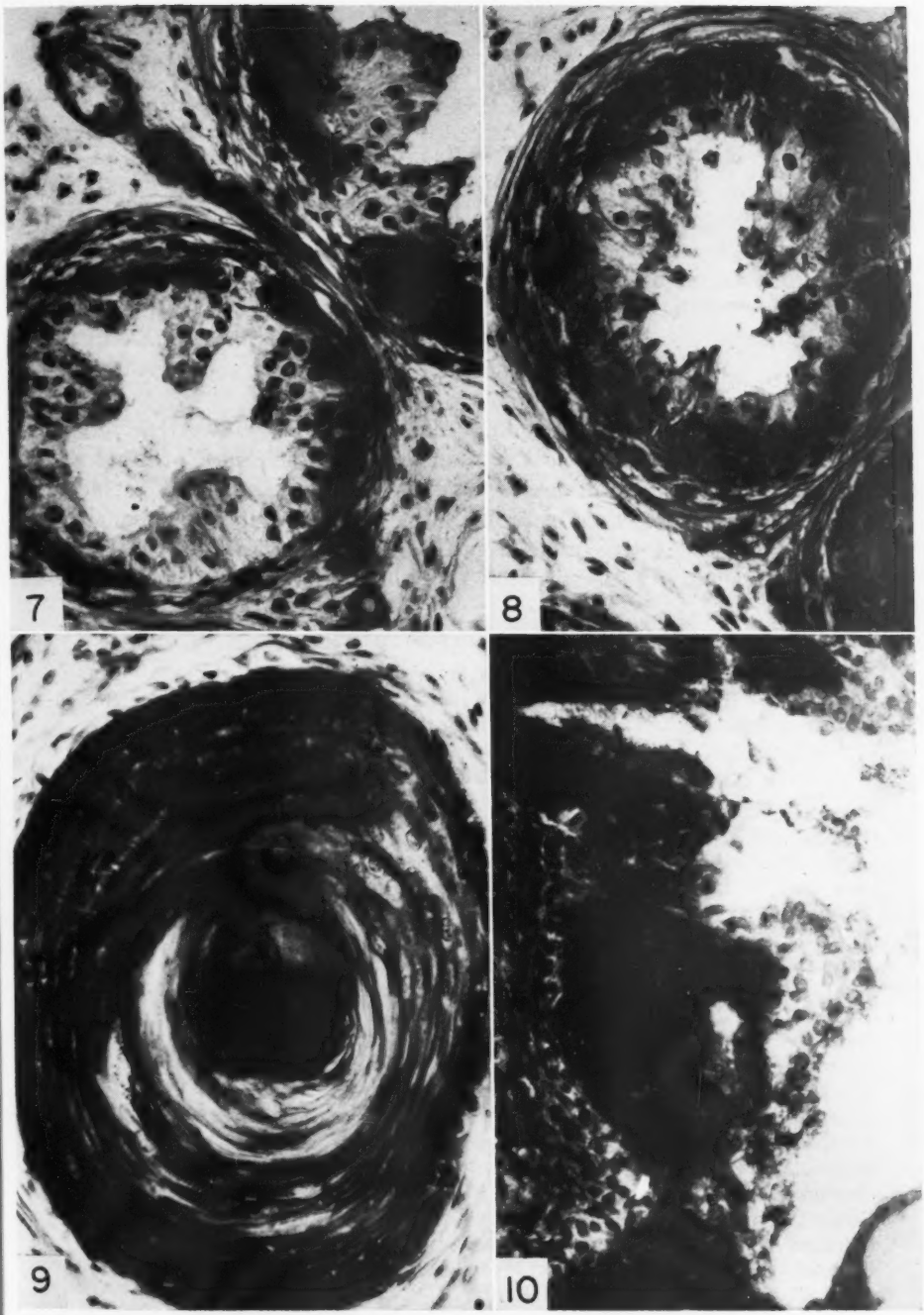

PLATE II

the possibility of the participation of stromal cells in epithelial metaplasia should not be overlooked.

SUMMARY AND CONCLUSIONS

Castration of male mice results in partial disappearance of stromal alkaline phosphatase activity from the vas deferens, seminal vesicle, anterior and dorsal prostates. Retention of phosphatase occurs in the subepithelial stroma of the vas and seminal vesicle, where activity seems to be relatively independent of testicular androgen. Estrogen administration maintains the normal localization of stromal alkaline phosphatase in the latter organs of castrates and thus may substitute for androgen in these areas.

Squamous metaplasia, including keratinization, occurs extensively in the anterior prostate and to a limited extent in the dorsal prostate and seminal vesicle. The intensely alkaline phosphatase-active replacing epithelium, first evidenced as proliferating basal cells, stands out in sharp contrast to the inactive original epithelium. In this process, the alkaline phosphatase activity serves almost as a differential stain.

Acknowledgments

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TESTOSTERONE STIMULATION OF SEMINAL VESICLES IN PTEROYLGLUTAMIC ACID-DEFICIENT RATS[1]

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IT HAS been demonstrated by Hertz (1945) and confirmed by Kline and Dorfman (1948) that pteroylglutamic acid (PGA) is essential to estrogen stimulation of the chick oviduct. This vitamin-hormone relationship has been extended to include estrogen-dependent tissues of other species, including the rodent (Hertz, 1948). In view of this relationship between PGA and estrogen it became of interest to investigate whether or not this vitamin is necessary to the biological effectiveness of androgens.

Conflicting reports have appeared in the literature concerning the action of testosterone propionate on accessory organs of male rats and mice deficient in PGA. Brendler (1949) found in castrate adult or intact immature male rats that, while α-estradiol inhibition of the prostate was interfered with by treatment with aminopterin, a specific antagonist to PGA, testosterone stimulation of the prostate was not thus interrupted. Brendler, however, has not indicated the basis on which he determines deficiency in PGA, since the data he presents do not show a significant limitation in body weight in the antagonist-treated rats.

Goldsmith, Black and Nigrelli (1949) demonstrated a decreased response of seminal vesicles and coagulating glands to testosterone propionate in castrate mice made deficient in pteroylglutamic acid by means of specific antagonists. A 1950 report by Goldsmith and Nigrelli, however, states that "the accessory genitalia in testosterone propionate treated castrate animals, both on an absolute and on a mg./100 gm. of body weight basis, were significantly lighter in mice on a restricted diet." They continue, "It would thus appear that the response of the accessory sex organs in the castrate mouse to a sufficient supply of male sex hormone is governed by an adequate food intake." In a personal communication, Goldsmith has stated, "It would thus appear to us at this time that the reduced growth response of the accessories may be due not to a lack of of folic acid *per se* but rather to the concomitant inanition" and, "The weight loss is undoubtedly a very decisive factor."

In brief, Brendler reports no change in effectiveness of testosterone in

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[2] Sigma Delta Epsilon Fellow, 1949–1950.

aminopterin-treated rats, which, however, show no significant decrease in body weights in comparison to body weights of the controls. Goldsmith's group, on the other hand, reports a decrease in effectiveness of testosterone in mice treated with PGA antagonists. Presumably the antagonist-treated mice are lower in body weight than the controls since the decreased effect of testosterone in animals suffering from inanition is cited as a probable explanation of its decreased action in the antagonist-treated animals. These data have been published only in the form of a condensed summary. Comparisons and statistical evaluation can, therefore, not be made.

An early attempt in our laboratory (1947) to discover whether or not a relationship exists between androgen action and deficiency in PGA seemed to indicate that testosterone propionate stimulation of seminal vesicles of castrate male rats was independent of PGA. These results were not published since data on inanition controls were not available. Repetition of the work with inclusion of inanition controls but using immature rats has only recently been done.

Other studies already submitted for publication have investigated the relationship of vitamin deficiencies to androgen action on the chick oviduct and chick comb. Androgen stimulation of the oviduct, but not of the comb, is dependent upon availability of PGA.

TABLE 1. PREVENTION OF THE ACTION OF AMINOPTERIN BY PRIOR INJECTION OF HIGH DOSES OF PTEROYLGLUTAMIC ACID: EXPERIMENT 63

Groups	Oil controls			9 mg. testosterone priopionate		
	No. of rats	Mean body weight gm. ± S.E.[a]	Mean weight seminal vesicles[b] mg. ± S.E.	No. of rats	Mean body weight gm. ± S.E.	Mean weight seminal vesicles[b] mg. ± S.E.
Unlimited Food Intake	8	67.0±1.8	13.0±0.6	8	65.3±2.3	54.8±2.3
Unlimited Food Intake plus Pteroylglutamic Acid	8	69.0±1.4	13.2±0.7	8	70.3±2.2	60.3±2.0
Unlimited Food Intake plus Pteroylglutamic Acid plus Aminopterin	8	68.1±1.4	12.8±0.7	8	69.5±1.6	59.4±1.3
Limited Food Intake (Inanition)	10	45.0±1.1	9.9±0.3	11	44.2±1.5	40.1±1.9

[a] Standard Error of the Mean.
[b] Weights given include coagulating glands.

MATERIALS[3] AND METHODS

Experiment 63, Table 1.—Four groups of Sprague-Dawley, immature, male rats were set up as indicated in Table 1, the day after the animals had been received in the laboratory. The aminopterin-treated group was given one subcutaneous injection of 20 μg. of aminopterin in 0.2 cc. of pyrogen-free, distilled water on the first day and 10 μg. in 0.1 cc., on the second day. The two groups treated with PGA received daily, 7.5 mg. in 0.5 cc. of solution, subcutaneously, for three successive days at the beginning of the experimental period. The injection of the PGA was always made one hour before the

[3] Generous amounts of pteroylglutamic acid and aminopterin were supplied by the Lederle Laboratories Division of the American Cyanamide Company. Testosterone propionate was kindly provided by Ciba Pharmaceutical Products, Inc.

injection of aminopterin in the group which received both compounds, care having been taken that the two injections were made at widely separated sites. All animals were allowed food[4] *ad libitum* except for the inanition controls which were rationed approximately 3 grams per day after the first day.

Testosterone propionate, 3 mg. in 0.1 cc. of corn oil, was administered subcutaneously daily for three days, beginning the third day of the experimental period to one half of each group of animals. The other half of each group received 0.1 cc. of oil, only.

The animals were sacrificed twenty-four hours after the third injection of hormone, on the sixth day of the experimental period, or at approximately thirty days of age.

Experiment 65, Table 2.—Immature, male rats of the Sprague-Dawley strain, on the day following their arrival in the laboratory, were separated into three groups as indi-

TABLE 2. THE ACTION OF TESTOSTERONE PROPIONATE IN AMINOPTERIN-TREATED IMMATURE RATS: EXPERIMENT 65

Groups	Oil controls			12 mg. testosterone propionate		
	No. of rats[a]	Mean body weight gm. ± S.E.	Mean weight seminal vesicles[b] mg. ± S.E.	No. of rats[a]	Mean body weight gm. ± S.E.	Mean weight seminal vesicles[b] mg. ± S.E.
Unlimited Food Intake	8 (0)	62.4±1.4	12.8±0.6	8 (0)	64.5±2.5	75.8±2.8
Aminopterin-Treated	9 (6)	39.2±1.1	7.6±0.7	13 (2)	39.3±2.5	56.2±2.8
Limited Food Intake (Inanition)	8 (0)	39.9±0.7	9.4±0.6	8 (0)	40.4±1.5	56.4±3.9

[a] No. of deaths indicated in parentheses.
[b] Weights given include coagulating glands.

cated in Table 2. The aminopterin-treated group was injected subcutaneously with 20 μg. of aminopterin in 0.2 cc. of pyrogen-free, distilled water. Of the other two groups, one was designated to serve as inanition controls and allowed approximately 3 gm. of food[4] per animal per day until the last day, when only 1 gm. per animal was permitted. All other animals had free access to food.

Beginning twenty-four hours after the injection of aminopterin, half of the animals in each of the three groups received testosterone propionate in corn oil and the other half, corn oil only, by subcutaneous injection daily for four days. Each dose of testosterone propionate was 3 mg. contained in 0.1 cc. of oil.

Autopsy was performed twenty-four hours after the fourth injection of hormone on the sixth day of the experimental period, when the animals were approximately thirty days of age.

EXPERIMENTAL RESULTS

In Experiment 63 it has been demonstrated that the decrease in body weight and in the response of seminal vesicles to testosterone propionate in aminopterin-treated, immature rats can be prevented by administering high doses of PGA before injecting the aminopterin. The experiment was set up to include groups treated with aminopterin only, but their mortality rate was so high that too few survived to autopsy to provide reliable data. None of the PGA-treated animals succumbed, however.

In Experiment 65, when only one injection of aminopterin (20 ug.) was given, a definite and severe PGA-deficiency developed, and yet enough

[4] Friskies, Dog Food Cubes; made by the Albers Milling Company, Sullivan, Wisconsin.

animals survived the treatment to provide adequate autopsy data. That a deficiency of PGA was produced was obvious in the severe diarrhea, anorexia, and weakness of the aminopterin-treated animals.

That the limitation of body growth in both the aminopterin-treated groups and in the inanition controls should eventuate in nearly identical average body weights in all four groups was fortuitous but advantageous in that speculation concerning the relationship of seminal vesicle weight to body weight is thereby eliminated. Comparison of the mean body weight of the normal animals with that of the aminopterin-treated rats and that of the inanition controls shows that there was a severe limitation of growth

TABLE 3. STATISTICAL ANALYSIS OF DATA FROM EXPERIMENT 65

Groups compared	Basis of comparison	t	P
Unlimited food (oil) and Aminopterin-treated (oil)	Body weight	12.96	<0.001
Unlimited food (T.P.*) and Aminopterin-treated (T.P.)	Seminal Vesicle Weight	4.9	<0.01
Unlimited food (oil) and Aminopterin-treated (oil)	Seminal Vesicle Weight	8.4	<0.01
Unlimited food (oil) and Inanition Controls (oil)	Seminal Vesicle Weight	4.3	<0.01
Aminopterin-treated (oil) and Inanition Controls (oil)	Seminal Vesicle Weight	3.0	0.01

* T. P. means injected with Testosterone Propionate

in both of the last two cases. The difference in mean body weight in each instance has a high statistical significance; for example, the difference between the mean body weights of the normal and the aminopterin-treated animals (oil injected) has a t-value of 12.96 and a P value of <0.001.

The data from Experiment 65 also show, that, although the aminopterin-treated rats were unable to respond as completely to a 12 mg. dose of testosterone propionate as did the normal animals they did respond as well to such stimulation as did the inanition controls with respect to absolute seminal vesicle weights. The decreased response in the PGA-deficient animals may, therefore, be accounted for entirely on the basis of the concomitant inanition.

Twelve milligrams of testosterone propionate in four equal daily doses brought about a six-fold increase in weight of seminal vesicles plus coagulating glands in the normal animals, a seven-fold increase in the aminopterin-treated animals and a six-fold increase in the inanition controls. This also may be interpreted as evidence that a deficiency of PGA does not interfere with the action of testosterone propionate on seminal vesicles.

The statistical analysis of the data shows that in the oil-treated controls there is a significant difference in seminal vesicle weight between the nor-

mal animals and the inanition controls; in addition, the difference in seminal vesicle weight of the inanition controls and the aminopterin-treated animals is also significant. These differences may be explained on the basis of decreased function of the pituitary gland (Moore and Samuels, 1931; Lutwak-Mann and Mann, 1950).

Aminopterin has been shown to have the effect of inciting adrenal hyperplasia (Higgins, 1949). If adrenal hyperplasia occurred in the aminopterin-treated rats of this experiment, it was not accompanied by increased production of androgens since the aminopterin-treated, oil-injected controls had a lower seminal vesicle weight than did the oil-injected inanition controls. Although inanition has been implicated as a stressing agent in adrenal studies, Cameron and Carmichael (1946) have indicated that enlargement of the adrenals in the rat occurs only in the advanced stages of acute starvation, and that this increase in size is not a true physiological growth but is rather a pathologic change involving increased water content. This has been confirmed by D'Angelo, Gordon and Charipper (1948).

SUMMARY

Testosterone propionate is less effective in stimulating the seminal vesicles of rats deficient in pteroylglutamic acid than those of normal rats. The reduced responsiveness to the hormone seen in the pteroyl-glutamic-acid-deficient rats is, however, explained by the concomitant inanition rather than by the vitamin deficiency of itself. The action of testosterone propionate on immature rat seminal vesicle weight, therefore, is not dependent upon pteroylglutamic acid.

NOTE: After this paper had been submitted for publication our attention was drawn to published data of Goldsmith and Nigrelli (1950) concerning the effect of inanition on the response to testosterone of accessory genital organs of castrate mice.

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TESTOSTERONE STIMULATION OF THE OVIDUCT IN VITAMIN-DEFICIENT CHICKS[1]

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A STUDY of the inter-relationships of vitamin deficiencies, inanition, and estrogen stimulation of the chick oviduct has been reported in a previous paper (Kline and Dorfman, 1950, a). Since it was shown that animals suffering from inanition manifest a nonspecific resistance to hormonal stimulation, oviduct responses to estrogen in vitamin-deficient animals were therefore interpreted by comparison to the response of inanition controls in order to correct for the inanition which inevitably accompanies vitamin-deficiency states. Of the four vitamin-deficiency states investigated, only that of a deficiency of pteroylglutamic acid (PGA) resulted in a severe limitation of oviduct response to estrogen stimulation. This confirmed the findings of Hertz (1945).

Since androgens are also capable of stimulating growth of the chick oviduct, a similar investigation of the inter-relationships of vitamin deficiencies, inanition and oviduct response to testosterone propionate has been made. The results indicate that oviduct proliferation in response to testosterone propionate, as to estrogens does not occur in the absence of PGA from the diet. In the other vitamin-deficiency states investigated such hormonally induced oviduct growth is not limited more than can be explained on the basis of the concomitant inanition alone. Studies to be reported in other papers have dealt with the relationship of vitamin deficiencies to the androgenic action of androsterone and testosterone on the chick comb and rat seminal vesicles (Kline and Dorfman, 1950, b and c).

MATERIALS AND METHODS

White Leghorn pullets, obtained from the Kerr Chickeries, Frenchtown, New Jersey, were received in the laboratory twenty-four to forty-eight hours after hatching and were maintained on the same synthetic ration[3] used in the experiments previously described (Kline and Dorfman, 1950, a). Inanition was produced by limiting the basal ration but

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[2] Sigma Delta Epsilon Fellow, 1949–1950.

[3] The PGA used in the diet was generously provided by the Lederle Laboratories Division of the American Cyanamid Co.

TABLE 1. OVIDUCT RESPONSE OF PGA[1]-DEFICIENT CHICKS TO TESTOSTERONE PROPIONATE

Experiment No.	Age at autopsy, days	Diet	Oil controls			3.0 mg. Testosterone propionate			Mean oviduct weight difference	
			No. of chicks	Mean body weight gm. ±S.E.[2]	Mean oviduct weight mg. ±S.E.	No. of chicks	Mean body weight gm. ±S.E.	Mean oviduct weight mg. ±S.E.	t[3]	(P)
1	25	Complete Synthetic	11	140 ±6.7	21 ±4.3	8	147 ±9.9	31 ±3.5	1.87	(0.1–0.05)
		Minus PGA	9	90 ±4.9	15 ±3.1	8	103 ±5.4	19 ±2.1	1.08	(0.3)
2	23	Complete Synthetic	9	131 ±5.7	20 ±1.0	8	120 ±4.8	42 ±2.5	8.20	(<0.01)
		Minus PGA	5	75 ±7.9	13 ±1.7	4	77 ±6.9	21 ±3.2	2.19	(0.1–0.05)
3	17	Complete Synthetic	10	93 ±6.7	14 ±1.0	10	90 ±4.0	30 ±2.9	4.02	(<0.01)
		Minus PGA	9	66 ±5.1	12 ±0.9	5	64 ±5.1	14 ±1.7	1.07	(0.2–0.1)

[1] Pteroylglutamic acid.
[2] Standard error of the mean.
[3] Fischer's t value.

not the B-vitamin supplement. Vitamin deficiencies were induced by omission of a single vitamin from the diet except in Experiment 50, in which the animals designated as deficient in PGA not only had been deprived of PGA but also had been fed a basal ration containing 1% of the crude antagonist[4] to PGA.

In Experiments 1, 2 and 3 (Table 1), the animals were placed on their respective diets between the second and fourth day of age and kept on these diets from fourteen to twenty-one days. Testosterone propionate[5] was injected subcutaneously once daily for six days, the daily dose of 0.5 mg. having been contained in 0.1 cc. of corn oil. Controls received corn oil only. Autopsy was performed twenty-four hours after the last injection.

In Experiment 50 (Table 3) a total of 8.7 mg. of testosterone propionate in 0.4 cc. of corn oil was injected subcutaneously in four equal daily doses. Autopsy was performed forty-eight hours after the last injection, when the animals were eighteen days of age.

RESULTS

In Experiments 1, 2 and 3 (Tables 1 and 2) it was demonstrated that 3 mg. of testosterone propionate produce a significant increase in oviduct

TABLE 2. STATISTICAL ANALYSIS OF DATA PRESENTED IN TABLE 1

Exp. No.	Testosterone propionate	Body weight		Oviduct weight	
		t	P	t	P
1	0	6.0	<0.01	1.2	0.30–0.20
	+	3.9	<0.01	3.0	0.01
2	0	5.7	<0.01	3.6	<0.01
	+	5.3	<0.01	5.1	<0.01
3	0	3.2	<0.01	1.5	0.20–0.10
	+	4.0	<0.01	4.8	<0.01

weight in pullets reared on a complete synthetic diet but is unable to cause tissue proliferation in the oviduct if PGA is omitted from the diet. Since the PGA-deficient chicks were also suffering from inanition, their body weights having been only two-thirds as great as those of the animals on the complete diet, the possibility that the absence of a significant response to the testosterone propionate was not due to the inanition alone was not eliminated.

In Experiment 50, animals deficient in PGA were compared with respect

[4] This substance represented the products of the condensation of 2,4,5-triamino-6-hydroxy-pyrimidine and p-amino-benzoyl-l(+)-glutamic acid with 2,3-dibromobutyraldehyde (Angier *et al.*, 1946; Franklin *et al.*, 1947). It was supplied to us by Dr. A. D. Welch of the Department of Pharmacology from material furnished by the Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

[5] Testosterone propionate was kindly supplied by Ciba Pharmaceutical Products, Inc.

to response of the oviduct to a high dose of testosterone propionate, to animals on an unlimited intake of the complete synthetic diet, to animals deficient in nicotinic acid or riboflavin, and to inanition controls (animals on a limited intake of the basal ration of the complete synthetic diet). Inanition decreased the response of the oviduct to the hormone, the re-

TABLE 3. OVIDUCT RESPONSE OF VITAMIN-DEFICIENT CHICKS TO TESTOSTERONE PROPIONATE: EXPERIMENT 50

Diet		Oil controls			8.7 mg. Testosterone propionate			Mean oviduct weight difference	
		No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	No. of chicks	Mean body weight gm. ± S.E.	Mean oviduct weight mg. ± S.E.	t	P
Complete Synthetic Diet	Unlimited Food Intake	11	95 ± 8	24 ± 5	11	88 ± 5	44 ± 3	3.3	<0.01
	Limited Food Intake (Inanition)	13	58 ± 3	16 ± 1	11	68 ± 3	35 ± 4	4.9	<0.01
	Minus PGA*	12	59 ± 3	19 ± 2	10	61 ± 3	22 ± 2	1.0	>0.05
	Minus Nicotinic Acid	8	60 ± 4	15 ± 2	10	68 ± 4	38 ± 3	3.3	<0.01
	Minus Riboflavin	9	56 ± 2	17 ± 1	9	59 ± 3	27 ± 3	3.4	<0.01

* Pteroylglutamic acid.

TABLE 4. STATISTICAL EVALUATION OF OVIDUCT WEIGHTS OF ANIMALS* RECEIVING TESTOSTERONE PROPIONATE: EXPERIMENT 50

Comparison of inanition to:	Mean oviduct weight difference	
	t	P
Minus PGA	3.1	<0.01
Minus Nicotinic Acid	—	>0.05
Minus Riboflavin	1.7	0.1

* Groups designated according to diet.

sponse having been correlated with body weight ($r = 0.740$; $P = 0.01$). As might be expected because of the concomitant inanition, the vitamin-deficient animals also showed a decreased response to the hormone in comparison to the animals given the complete diet in unlimited amounts. The PGA-deficient animals, however, were the only vitamin-deficient animals which showed a responsiveness diminished beyond that which can be accounted for on the basis of inanition alone. Statistical analysis of the data (Table 4) shows that the pullets deficient in nicotinic acid or in riboflavin had oviduct weights not significantly different from those of the animals suffering from inanition alone. The difference in mean oviduct weights of the PGA-deficient pullets and the inanition controls was highly significant ($P < 0.01$).

DISCUSSION

Pteroylglutamic acid (PGA) is necessary to proliferation of chick oviduct tissue in response to both estrogenic and androgenic stimulation. Tissue proliferation in general, however, is not necessarily dependent upon

the presence of PGA. For instance, the growth of seminal vesicles in PGA-deficient rats in response to testosterone propionate is not limited more than in inanition controls (Kline and Dorfman, 1950, b). All phases of the biological action of estrogen are, on the other hand, not necessarily dependent upon the presence of PGA since mobilization of fat can occur in response to stilbestrol in PGA-deficient animals (Hertz, 1948). Furthermore, a deficiency of riboflavin or of nicotinic acid does not interfere with oviduct response to testosterone propionate. The extreme limitation of oviduct response to either androgen or estrogen stimulation in animals deficient in PGA would therefore seem to be due to the requirement of PGA as an essential for oviduct tissue proliferation, rather than as an essential for tissue growth or hormonal action in general.

The possibility still remains, of course, that the ability of testosterone propionate to effect oviduct growth depends upon its biological conversion to estrogen (Dorfman, 1948). If this be its mode of exhibiting estrogenic activity, a dependence on PGA would be expected. If, on the other hand, testosterone does stimulate oviduct growth without molecular transformation to estrogen, the evidence suggests that it functions in the same enzyme systems as do estrogens.

SUMMARY

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SUMMARY

Pteroylglutamic acid is essential to proliferation of chick oviduct tissue in response to stimulation by testosterone propionate. The diminished response of the oviduct to testosterone propionate observed in chicks deficient in nicotinic acid or riboflavin, however, can be explained on the basis of a nonspecific resistance to stimulation due to the concomitant inanition.

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STUDIES ON THE RELATIONSHIP OF THE ADRENAL CORTEX TO THE EXPERIMENTAL PRODUCTION OF HYPOFERREMIA IN RATS[1]

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IN A previous paper (Cartwright, Hamilton, Gubler, Fellows, Ashenbrucker and Wintrobe, to be published) in this series the results of experiments in dogs were reported which were designed to elucidate the mechanism by which hypoferremia is produced. It was found that a variety of agents was capable of producing an acute hypoferremia in dogs. These included bacterial abscesses, sterile turpentine abscesses, histamine, epinephrine, fracture, anaphylactic shock and occasionally the stress of taking blood samples. Adrenal cortical extract and the adrenocorticotrophic hormone (ACTH) were found to produce hypoferremia to a similar degree. In the adrenalectomized dog the hypoferremia caused by mild stress and by ACTH was abolished; the hypoferremic effect of epinephrine was significantly decreased but was not abolished. Intravenously administered saccharated oxide of iron was found to be cleared most rapidly from the plasma of intact dogs given ACTH and most slowly from the plasma of adrenalectomized dogs, the rate of clearance in the intact controls falling in between.

Since it has been found that the plasma iron is diverted to the liver and spleen (Greenberg, Ashenbrucker, Lauritsen, Worth, Humphreys and Wintrobe, 1947), and that the hypoferremia is prevented by the administration of colloidal thorium dioxide (Cartwright, Gubler and Wintrobe, 1950), it was postulated that the level of plasma iron is regulated by the functional state of the reticulo-endothelial system and that the adrenal cortex stimulates the reticulo-endothelial system to take up iron from the plasma. This explanation is admittedly speculative, however, and other theories must be considered. For example, it is possible that ACTH and cortisone produce hypoferremia through an entirely different mechanism than does turpentine. This is suggested by the fact that epinephrine and turpentine produce some degree of hypoferremia in adrenalectomized

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dogs. Whether or not this is by direct stimulation of the reticulo-endothelial system is unknown.

The studies reported in this investigation were undertaken to determine whether the observations obtained in dogs could be confirmed in rats.

METHODS

In these studies approximately 500 male Sprague-Dawley rats weighing between 100 and 200 grams were used. The animals were fed Purina Dog Chow and allowed water *ad libitum*.

Bilateral adrenalectomy was performed by the dorsal route. The completeness of adrenalectomy was confirmed at postmortem. Following adrenalectomy the rats were continued on the Purina Dog Chow diet and were given a 0.9 per cent solution of sodium chloride to drink. Adrenalectomized rats were used for experimental procedures three days after operation, it having been shown in previous experiments that the plasma iron remains constant for a period of between 3 days to 3 weeks after adrenalectomy.

To minimize the effects of variations in the plasma iron which occur in different batches of Sprague-Dawley rats, a control group was taken from each batch for each experimental procedure. To evaluate the effects of the operative procedure on the level of plasma iron a sham operation was performed on groups of rats and these were used as controls and for experimental procedures for comparison with intact and adrenalectomized animals. The sham operation consisted of mobilizing the adrenals via the dorsal approach and then replacing them. Sham operated rats were used for experimental procedures three days after operation. An additional group of rats was splenectomized as a further type of operative control.

All glassware used for the collection and storage of blood samples and also for the determination of plasma iron was carefully cleaned with acid and washed three times with double distilled water. For plasma iron determinations rats were anesthetized with ether, and blood was collected from the abdominal aorta in all glass heparinized syringes and placed in 15 ml. centrifuge tubes to which 2 drops of 20 per cent potassium oxalate had previously been added. The plasma was then separated by centrifugation and the iron estimation made by a modification (Hamilton, Gubler, Cartwright and Wintrobe, to be published) of the method of Barkan and Walker (1940) using the Beckman spectrophotometer.

In the previous paper (Cartwright *et al.*, to be published) the standard error of a single determination of the plasma iron was calculated for the methods used. This was found to be ± 3.7 μg. per cent.

It was not possible to make serial plasma iron determinations on an individual rat. Therefore, rats were used in groups and single determinations were made on each individual rat. The data have been treated statistically and the mean value of the plasma iron of each group and the standard error of the mean have been calculated. The "t" test has been used to estimate the significance of differences between group.

RESULTS

The effect of operation and adrenalectomy on the plasma iron. Data for the plasma iron of intact and sham operated rats are presented in Table 1. The values for the intact rats are in good agreement with previous levels reported from this laboratory (Wintrobe, Grinstein, Dubash, Humphreys,

Ashenbrucker and Worth 1947). The sham operated rats were sacrificed three days following operation and it can be seen that the level of plasma iron was lower than that of the intact rats. This difference was not significant ($P = > .05$) and therefore the two groups have been combined to form a single control group.

Table 1 also gives the plasma iron of adrenalectomized rats sacrificed three days after operation. The level of plasma iron in the adrenalectomized group was significantly lower than that of the control group ($P = < .001$). Thus it appears that, in the rat, adrenalectomy causes a decrease in the level of plasma iron. The failure of the sham operation and

TABLE 1. THE LEVEL OF PLASMA IRON IN INTACT, SHAM OPERATED, ADRENALECTOMIZED AND SPLENECTOMIZED RATS

Group	Number of rats	Plasma iron μg. %	S.E.
Intact	29	282	±16
Sham Operated	39	246	±12
Intact and Sham Operated	68	261	±13
Adrenalectomized	59	155	± 6
Splenectomized	10	267	±15

S.E. refers to standard error of the mean

of splenectomy to cause a significant fall in the plasma iron indicates that the operative procedure itself is not the factor responsible for this decrease.

To evaluate whether the fall in plasma iron following adrenalectomy might be due to reduced food intake or reduced absorption of iron, 80 rats were kept on an iron low diet[3] for 26 days. Of these 80, 20 rats were on restricted intake of 5 grams of diet daily, 20 were fed *ad libitum* and 40 were adrenalectomized on the tenth day of the diet. All rats were given 0.5 mg. ferrous chloride intramuscularly every other day for 6 doses between the 14th and 25th of the diet, making a total of 3.0 mg. of iron, and samples for plasma iron determinations were taken 24 hours after the last injection of iron on the 26th day of the diet. The plasma iron of the 20 *ad libitum*-fed rats was 179 ± 7, that of the 18 diet-restricted rats 160 ± 8 and that of 33 adrenalectomized rats 104 ± 5. There was no significant difference between the levels in the rats fed *ad libitum* and in those whose diet was restricted. There was, however, a significant difference between the control and the adrenalectomized groups ($P = < .001$). These results suggest that though food intake and absorption of iron may be reduced following adrenalec-

[3] The diet consisted of crude casein (Sheffield "New Process") 27 per cent, sugar 56 per cent, lard 7 per cent, salt mixture 4 per cent, cod liver oil 2 per cent and powdered yeast 4 per cent. The salt mixture had the following percentage composition: $NaHCO_3$ 27.4, $CaHPO_4$ 25.0, $MgSO_4$ 14.0, K_2CO_3 4.65, KCl 31.2, $CuSO_4$ 0.24, $MnSO_4$ 0.15, KI 0.15 and NaF 0.03.

tomy, these are not the significant factors in the decrease of plasma iron which follow adrenalectomy.

The effect on the plasma iron of supporting adrenalectomized rats with adrenal cortical extract, cortisone or desoxycorticosterone acetate. Thirty rats were divided into three groups. One group of 10 was kept as a control group. The remaining twenty rats were adrenalectomized and 10 of these received intramuscularly 0.1 ml. of lipo-adrenal cortical extract daily for the first three days postoperatively. All animals were sacrificed on the same day and blood samples were taken for plasma iron. The results are given in Table 2. It can be seen that there was a significant difference between the

TABLE 2. THE LEVEL OF PLASMA IRON IN CONTROL AND ADRENALECTOMIZED RATS, AND IN ADRENALECTOMIZED RATS SUPPORTED WITH ADRENOCORTICAL EXTRACT, CORTISONE OR DESOXYCORTICOSTERONE ACETATE

Group	Number of rats	Plasma iron μg. %	S.E.
Control	10	258	±13
Adrenalectomized & Cortical Extract	10	257	±12
Adrenalectomized	10	163	± 9
Control	10	291	±23
Adrenalectomized & Cortisone	9	275	±15
Adrenalectomized & Desoxycorticosterone Acetate	10	199	±17
Adrenalectomized	10	173	±10

S.E. refers to standard error of the mean.

plasma iron of the control and that of the adrenalectomized groups ($P = <.001$); there was no difference between the plasma iron of the control group and that of the adrenalectomized group supported with lipo-adrenal cortical extract.

Four additional groups of rats were arranged as indicated in Table 2. Twenty-nine rats were adrenalectomized; of these 9 were given 0.25 mg. of cortisone intramuscularly daily for the three days post operatively, 10 were given 0.5 mg. of desoxycorticosterone acetate daily for the same period and 10 received no supportive treatment. The animals were all sacrificed on the third day postoperatively and blood samples for plasma iron were taken. The results (Table 2) show that there was a significant difference between the level of plasma iron in the intact group and that of the adrenalectomized untreated group ($P = <.001$). There was also a significant difference between the level of iron in the control group and that of the adrenalectomized group given desoxycorticosterone acetate ($P = <.01$) and, as a corollary to this, there was no difference between the latter group and the untreated adrenalectomized group ($P = >.1$). There was no difference between the level of iron in the intact control group and that of the adrenalectomized group supported with cortisone

(P = >.5) but there was a significant difference between the level of iron of the latter group and that of the untreated adrenalectomized group (P = <.001).

The effect of adrenocorticotrophic hormone on the plasma iron of sham operated and adrenalectomized rats. A single injection of adrenocorticotrophic hormone was given to 8 sham operated rats and to 8 adrenalectomized rats. Of these, four in each group received a dose equivalent to

TABLE 3. THE HYPOFERREMIC EFFECT OF A VARIETY OF AGENTS IN INTACT, SHAM OPERATED AND ADRENALECTOMIZED RATS

Substance injected	Group	Control			Injected			Decrease P.I. μg. %	Per cent Decrease in P.I.	P
		Number rats	P.I. μg. %	S.E.	Number rats	P.I. μg. %	S.E.			
A.C.T.H. 5–10 mg.	Sham Op.	10	230	±19	8	113	±15	117	51	<.001
	Adrenalectomized	10	157	±17	8	166	±21	0	0	>.7
C.E. 2.0 ml.	Sham Op.	10	256	±30	9	92	± 4	164	62	<.001
	Adrenalectomized	10	163	± 9	9	100	±10	63	39	<.001
C.E. 0.5 ml.	Intact	9	274	±15	10	167	± 9	107	39	<.001
Cortisone 4.0 mg.	Intact		258	±13	10	180	±22	78	31	<.01
Cortisone 8.0 mg.	Intact	10	291	±23	10	188	± 8	103	35	<.001
D.C.A. 5.0 mg.	Intact	9	258	±13	10	187	± 9	71	28	<.001
Turpentine 0.1 ml./100 Gm.	Intact	10	293	±36	10	89	± 9	204	70	<.001
	Adrenalectomized	9	177	±15	10	75	± 5	102	58	<.001
Epinephrine 100γ/100 Gm.	Sham Op.	10	245	±17	9	223	±25	22	9	>.4
	Adrenalectomized	10	139	± 9	10	95	± 5	44	32	<.01
Histamine 2.0 mg./100 Gm.	Sham Op.	9	254	±23	9	213	±15	41	16	<.02>.01
	Adrenalectomized	10	122	±15	9	108	±11	14	11	>.4
Histamine 10.0 mg./100 Gm.	Sham Op.	10	243	±19	9	198	±11	45	19	>.1
	Adrenalectomized	10	140	±12	7	114	±20	26	19	>.1
Histamine 100 mg./100 Gm.	Intact	9	274	±15	9	143	±14	131	48	<.001

A.C.T.H., adrenocorticotrophic hormone; C.E., lipo-adrenal cortical extract; D.C.A., desoxycorticosterone acetate; P.I., plasma iron; S.E., standard error of the mean; P, probability.

5 mg. of Armour's standard (LA-1-A), and the remaining four were given a dose equivalent to 10 mg. of Armour's standard. There was no correlation between the dose given and the effects on the plasma iron and therefore each group will be considered as a whole. Samples were taken for plasma iron determinations from 10 untreated sham operated and 10 untreated adrenalectomized rats which had been operated on at the same time as the injected rats and were from the same batch of rats. This procedure was followed in evaluating the effects of the injection of all agents. The injected rats were sacrificed 6 hours following the administration of ACTH. The results are given in Table 3. It can be seen that following the injection of ACTH there was a significant fall in the level of plasma iron in the sham operated rats. There was no decrease in the level of plasma iron of the adrenalectomized group following the injection of ACTH.

An additional group of ten intact rats was given a dose equivalent to 8 mg. of ACTH (LA-1-A) daily for 5 days. This dose was divided and given in 3 intramuscular injections at 9:00 A.M., 5:00 P.M. and 1:00 A.M. At each injection the rats received the ACTH dissolved in 0.5 ml. of 0.9 per cent saline. Samples were taken from the treated group 6 hours after the last injection of ACTH and also from a corresponding control group. The mean plasma iron level of the control group of 10 intact rats was 291 ± 23, while the mean plasma iron level of the 10 intact rats injected with ACTH was 194 ± 10. Thus the administration of ACTH over 5 days to intact rats produced a significant decrease in the plasma iron ($P = < .01$ and just $> .001$).

The effect of adrenal cortical extract on the plasma iron of sham operated and adrenalectomized rats. A single injection of 2.0 ml. of lipo-adrenal cortical extract was given intramuscularly to 9 sham operated rats and to 9 adrenalectomized rats. Samples were taken for plasma iron determinations six hours following the injections in these animals and from control groups of untreated sham operated and adrenalectomized rats. The results (Table 3) show that in the sham operated rats the injection of adrenal cortical extract caused a significant fall in the level of the plasma iron. It can be seen that though adrenalectomy itself produced a significant fall in the plasma iron ($P = < .001$), the injection of 2.0 ml. of adrenal cortical extract in adrenalectomized rats caused a further significant decrease in the level of plasma iron in six hours.

The effect of cortisone on the plasma iron of intact rats. A single intramuscular injection of 4.0 mg. of cortisone was given to 10 intact rats. This dose was chosen because 2.0 ml. of lipo-adrenal cortical extract is reported to contain the biological equivalent of 2.0 mg. of cortisone-like steroids. Samples were taken from the injected rats after 6 hours and samples were obtained from untreated controls at the same time. This dose of cortisone produced a significant decrease in the level of plasma iron in the intact rats (Table 3).

An additional group of 10 intact rats were given a single intramuscular injection of 8.0 mg. of cortisone. This dose was chosen because the decrease in the plasma iron produced by the injection of 4 mg. of cortisone was approximately half that produced by the injection of 2 ml. of lipo-adrenal cortical extract. Samples were taken from the injected rats after 6 hours and from controls at the same time. This dose of cortisone produced a significant decrease in the level of the plasma iron of intact rats (Table 3) but the decrease was no greater than with the 4 mg. dose of cortisone.

The effect of desoxycorticosterone acetate on the plasma iron of intact rats. A single injection of 5.0 mg. of desoxycorticosterone acetate was given intramuscularly to 10 intact rats. Samples were taken from these and from untreated controls after 6 hours. The injection of desoxycorticosterone

produced a significant decrease in the level of plasma iron of intact rats (Table 3).

The effect of turpentine on the plasma iron of intact and adrenalectomized rats. A single injection of 0.1 ml. of turpentine per 100 gm. of body weight (rectified oil of turpentine, Rexall, USP) was given intramuscularly to 10 intact and 10 adrenalectomized rats. Samples for plasma iron determinations were taken 24 hours after the injection and samples were taken at the same time from 10 untreated intact and 9 untreated adrenalectomized rats. In both the intact and adrenalectomized groups the injection of turpentine was followed at 24 hours by a significant fall in the level of the plasma iron (Table 3).

The effect of epinephrine on the plasma iron of intact and adrenalectomized rats. A single injection of 100 γ of epinephrine per 100 gm. of body weight (Parke, Davis & Co. aqueous solution) was given subcutaneously to 9 sham operated and 10 adrenalectomized rats. Samples for plasma iron determinations were taken 6 hours following the injection and at the same time samples were taken from untreated sham operated and adrenalectomized rats. In Table 3 it can be seen that this dose of epinephrine failed to produce any fall in the plasma iron of the sham operated rats, but produced a significant decrease in the plasma iron level of the adrenalectomized rats.

Since 100 γ of epinephrine failed to produce hypoferremia, 10 sham operated and 10 adrenalectomized rats were given a single injection of 500 γ of epinephrine per 100 gm. of body weight. Within ten minutes following the injection of the epinephrine the adrenalectomized rats began to experience respiratory difficulty which was accompanied by a bloody, frothy discharge from the nose and mouth. Respiration rapidly became irregular and the rats developed convulsions and all died within 20 minutes after the injection of epinephrine. Similar results were noted in the sham operated rats although the onset of respiratory difficulties occurred at between 15 and 20 minutes after the epinephrine, the frothy discharge and convulsions were less marked than in the adrenalectomized rats and death occurred in all but one within 30 minutes. The remaining rat survived 4 hours. These results are similar to those reported by Nickerson (Nickerson, Berghout and Hammerstrom, 1950) on the mechanism of the acute lethal effect of epinephrine in rats. No samples for plasma iron were taken from these rats.

The effect of histamine on the plasma iron of intact and adrenalectomized rats. Nine sham operated and 9 adrenalectomized rats were given a single injection of 2.0 mg. of histamine per 100 gm. of body weight intramuscularly. Samples for plasma iron determinations were taken 6 hours after the injection and at the same time samples were taken from untreated

sham operated and adrenalectomized rats. This dose of histamine appeared to produce a significant fall in the plasma iron of the intact rats but failed to have any effect on the level of iron of the adrenalectomized rats (Table 3).

An additional 9 sham operated and 9 adrenalectomized rats were given a single injection of 10.0 mg. of histamine per 100 gm. of body weight intramuscularly. Two of the adrenalectomized rats died during the day. Samples were taken from the remaining rats at 6 hours after the injection and from control groups of sham operated and adrenalectomized rats. This dose of histamine failed to produce any significant decrease in the plasma iron of the sham operated and adrenalectomized rats (Table 3).

A single injection of 100 mg. of histamine per 100 gm. of body weight was given intramuscularly to 9 intact rats. At approximately 10 minutes after the injection all the rats appeared to be very distressed and had obvious dyspnea. Thirty minutes later they appeared to have recovered completely from the effects of the histamine. Samples were taken 6 hours after the injection and at the same time samples were obtained from an intact untreated group. In Table 3 it can be seen that this dose of histamine produced a significant fall in the plasma iron level of intact rats.

DISCUSSION

These results in rats confirm and extend the findings in dogs which indicate that there is a relation between adreno-cortical function and the level of plasma iron (Cartwright *et al.*, to be published). It has been found in the rat as in the dog that the injection of adrenal cortical extract and of adreno-corticotrophic hormone produces acute hypoferremia. Furthermore, adrenalectomy in the rat, as in the dog, abolishes the hypoferremic effect of ACTH, indicating that this hormone mediates its hypoferremia-producing action via the adrenal cortex. In addition, it has been shown that cortisone can produce hypoferremia in the rat and that hypoferremia can be maintained for five days by the repeated injection of ACTH.

These studies also confirm the report of Majumder and Wintrobe (1948) that the injection of turpentine is followed by a significant decrease in the plasma iron of both intact and adrenalectomized rats, as well as the previous observations in dogs (Cartwright *et al.*, to be published) that severe stresses such as turpentine can produce hypoferremia in the absence of the adrenal cortex. In this respect it is interesting to note that epinephrine failed to produce hypoferremia in the intact rat in contrast to the invariable decrease in the plasma iron which followed its administration to dogs. In the rat, adrenalectomy appears to have made the rat more sensitive to the stress of epinephrine. The hypoferremia which occurs in the absence of the adrenal cortex is confirmatory of similar observations

made in the dogs. Doses of histamine approximately 10 and 50 times greater than the equivalent dose in the dog failed to produce a hypoferremia in any way comparable with that produced in the dogs by a much smaller dose. Appreciable hypoferremia in the intact rat only followed the injection of a dose of histamine approximately 500 times greater than that given to the dogs.

It is surprising that histamine in doses of 2 and 10 mg. per 100 gm. body weight failed to produce hypoferremia in adrenalectomized rats since it has been shown that the adrenalectomized rat has less ability than the intact rat to inactivate histamine (Rose, 1939). Presumably because of this it has been reported that both blood (Wilson, 1941) and tissue histamine (Rose and Browne, 1941) levels are elevated after adrenalectomy. This evidence suggests that histamine is not an intermediary factor common to all stressful agents in the production of hypoferremia.

The most important new evidence derived from these studies of the relation between the adrenal cortex and the level of plasma iron is the fact that adrenalectomy results in a significant decrease in the plasma iron. In the previous study in dogs it was noted that the mean initial plasma iron levels were all lower in the adrenalectomized groups than in the corresponding intact groups; but in only one adrenalectomized group was the initial iron level statistically significantly lower than in the control group. The dogs in this adrenalectomized group had been maintained largely on desoxycorticosterone acetate in contrast to the other adrenalectomized dogs which had been supported with small doses of adreno-cortical extract. In the present study it has been shown in rats that small doses of either adreno-cortical extract or of cortisone will prevent the decrease in the level of plasma iron following adrenalectomy and that desoxycorticosterone acetate is ineffective in this respect. These results afford an explanation of the previous findings in dogs and reveal a new aspect in the relation of adreno-cortical function to the level of plasma iron.

It appears that adreno-cortical steroids are essential for the maintenance of the normal level of plasma iron. Thus, in addition to the part the adrenal cortex plays in the production of acute hypoferremia following stress, adreno-cortical function is concerned with the homeostasis of plasma iron under the conditions of everyday existence. The apparent paradox that one dose of adreno-cortical hormone will produce a decrease in the level of plasma iron while another dose will maintain the plasma iron at its normal level emphasizes the role of the adrenal cortex as an organ of homeostasis as well as its ability to endow the organism with resistance to all types of stress. This capacity of adreno-cortical hormone is paralleled by its numerous and diverse actions in other types of metabolic processes. Thus adreno-cortical steroids may promote as well as inhibit glucose

utilization (Ingle, 1948; Ingle and Nezamis, 1948; Ingle, Prestrud, Nezamis and Kuizenga, 1947), and stimulate anabolism as well as catabolism (Ingle and Prestrud, 1949), depending upon the dose of steroid employed. As has been emphasized by Sayers, when the tissues are utilizing the hormone at a rate approximately equal to the supply, the metabolic effects of cortical steroids may be quite different from those observed when they are exposed to an excess of hormone. For example, in the regulation of electrolyte metabolism Woodbury *et al.* (1950) have presented evidence that adreno-cortical steroids can "normalize" plasma sodium regardless of the direction of the deviation from normal. Other examples of this normalizing effect have been presented in an excellent review by Sayers (1950).

It will be noted that while desoxycorticosterone acetate in a dose of 5 mg. produces a significant decrease in the plasma iron of the intact rat in six hours, it fails to maintain the normal level of plasma iron in the adrenalectomized rat in a dose of 0.5 mg. per day, in contrast to adreno-cortical extract and cortisone. It is significant in this connection that desoxycorticosterone acetate fails to substitute for the 11–17 oxysteroids in a number of other physiological conditions.

There are two possible mechanisms by which desoxycorticosterone acetate may produce acute hypoferremia. This steroid in the dose of 5 mg. may be acting as a non-specific stressful agent. Alternatively it may be producing a "chemical hypophysectomy" since it has been shown that desoxycorticosterone acetate produces the metabolic changes characteristic of adreno-cortical insufficiency. Thus, desoxycorticosterone acetate produces hypersensitivity to insulin (Cheng and Sayers, 1949), increased glucose tolerance in normal (Köhler and Fleckenstein, 1942, 1943) and diabetic subjects (Köhler and Wegener, 1949; Köhler and Fleckenstein, 1944) and a decrease in the rate of urinary nitrogen excretion (Ingle and Thorn, 1941; Conn, Johnston and Lewis, 1946). Furthermore it has been shown that the toxic actions of desoxycorticosterone acetate in rats are prevented by the administration of ACTH, adrenocortical extract (Woodbury, Rosenberg and Sayers, 1950) or cortisone (Woodbury and Sayers, personal communication). This is in agreement with the finding of Crafts and Walker (1947) that hypophysectomy results in a significant decrease in the plasma iron of rats.

SUMMARY

1. Adrenalectomy resulted in a significant decrease in the level of the plasma iron of rats. This decrease was prevented by the daily administration of small doses of adreno-cortical extract or cortisone but not desoxycorticosterone acetate.

2. Decreased food intake and decreased absorption of iron were not the significant factors in the decrease of plasma iron which followed adrenalectomy.

3. The injection of adreno-corticotrophic hormone, adreno-cortical extract, cortisone, desoxycorticosterone acetate, and turpentine produced acute hypoferremia in intact or sham operated rats.

4. Adrenalectomy abolished the hypoferremia-producing effect of adreno-corticotrophic hormone; adreno-cortical extract and turpentine produced a significant hypoferremia in the absence of the adrenal cortex.

5. Epinephrine failed to produce hypoferremia in sham operated rats but did so in adrenalectomized animals. Significant hypoferremia was produced in intact rats only by a very large dose of histamine.

6. The results in rats have thus confirmed the observation in dogs that there is a relationship between the adrenal cortex and the development of hypoferremia following stress, and also have revealed that the adrenal cortex plays an essential role in the maintenance of the normal homeostasis of the plasma iron level.

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THE ROLE OF HORMONES IN ADIPOSE TISSUE GLYCOGEN SYNTHESIS IN THE RAT: THE ADRENAL CORTEX

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KNOWLEDGE concerning the role of the adrenal cortex in fat metabolism is fragmentary and, in many cases, contradictory. This subject was reviewed by Ingle in 1943 and the present status of the problem may be summarized briefly as follows. The adrenalectomized animal does not readily mobilize fat from peripheral tissue to liver or develop ketosis in response to fasting or stress (Ingle, 1943) but may do both under the influence of growth hormone (Bennett *et al.*, 1948, Szego and White, 1949). Fat stores of the adrenalectomized rat allowed to eat *ad libitum* become depleted (Schiffer and Wertheimer, 1947), although Stoerk and Porter (1950) claim this occurs only in adrenalectomized rats on a restricted food intake. The carcasses of adrenalectomized rats contain more protein and less fat than pair-fed controls (Winternitz, 1942). Conversely, the administration of adrenocorticotrophic hormone or the C-11 oxygenated steroids results in an apparent rapid mobilization of fat from peripheral depots to the liver (Baker *et al.*, 1948), ketosis (Bennett *et al.*, 1948) and a fall in the respiratory quotient in the fasting organism (Thorn *et al.*, 1940). Kendall (1945) has reported marked storage of fat on chronic administration of cortical steroids to mice, while Li *et al.* (1949) have found an increase in the proportion of fat in the carcasses of hypophysectomized rats on administration of ACTH. Baker *et al.* (1948) have described disappearance of subcutaneous fat in ACTH treated animals. While in pancreatic diabetes Stetten and Klein (1945, 1946) have demonstrated that a defect in lipogenesis from carbohydrate is an outstanding characteristic, no comparable studies have been recorded as yet in ACTH or adrenal steroid diabetes. Spontaneous and induced adrenal cortical hyperfunction in the human, Cushing's syndrome, is characterized by a peculiar distribution of fat with an excess accumulation often taking place in the trunk, neck and face, while a loss occurs in the extremities. Whether an actual net increase in fat occurs in this condition is probably dependent on the food intake, but the distribution in any case appears to be determined by

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adrenal hormone. Any consideration of the role of the adrenal cortex in the metabolism of adipose tissue must take this last fact into consideration, i.e., the adrenal cortex may have different qualitative and quantitative effects on adipose tissue in different parts of the body.

In previous studies from this laboratory (Engel and Scott, 1950, Scott and Engel, 1950) the application of the study of glycogen synthesis in the dorsal interscapular fat body of the rat to the general problem of hormonal control of fat metabolism has been considered. It was pointed out that while Wertheimer and Shapiro (1948) have presented circumstantial evidence that the concentration of glycogen in adipose tissue may parallel conversion of carbohydrate to fat there is as yet no convincing proof that glycogen is a necessary intermediary in this conversion, either under normal circumstances or when there is a plethora of carbohydrate to be disposed of. So far, only in the case of insulin, which is known to be concerned with lipogenesis from carbohydrate, have data been accumulated (Wertheimer and Shapiro, 1948, Fawcett, 1947, Engel and Scott, 1950) which can be interpreted to support the interpretation of Wertheimer and Shapiro. The present report is concerned with the role of the adrenal cortex in fat glycogen synthesis. It will be shown that neither ACTH nor potent adrenal extracts or steroids have any consistent effect on adipose tissue glycogen concentration, but when given in conjunction with insulin significant increases in fat glycogen occur. The adipose tissue glycogen content of the fasted adrenalectomized rat is normal, but is lower than normal in fed adrenalectomized rats with or without insulin treatment.

MATERIALS AND METHODS

The design of the experiments, preparation of animals and analytical techniques were the same as in the previous reports (Engel and Scott, 1950, Scott and Engel, 1950) except where otherwise indicated.

The hormone preparations used were Upjohn's Adrenal Cortex Extract, from which the alcohol was distilled off in vacuo prior to use; Upjohn's Lipoadrenal Cortex and a batch of pooled assay samples of hog adrenal extract in oil prepared by the Upjohn Co.; Cortisone Acetate (Merck) as a suspension of microcrystals in saline; and ACTH prepared by Dr. George Sayers and by the Armour Co. The ACTH was generally made up in saline immediately prior to use and any left over kept in the frozen state. A highly purified sample of Lilly's insulin was used. In most experiments the entire dorsal fat body was removed, dissected free from surrounding white fat and weighed to the nearest tenth of a milligram on a torsion balance in order to determine whether any of the experimental procedures had any effect on the size of this structure.

RESULTS

Effects of Adrenal Cortex Extract and Lipoadrenal Cortex

Table 1 records the results of intensive treatment with Upjohn's Adrenal Cortex Extract (ACE) and Lipoadrenal Cortex (LAC) in 24 and 48 hour

fasted rats and 48 hour fasted rats given glucose by stomach tube. Values for dorsal adipose tissue glycogen, liver glycogen and blood sugar are recorded. Hourly administration of 1 ml. of ACE subcutaneously for 8 hours resulted in no change in the adipose tissue glycogen and blood sugar of the 24 and 48 hour fasted rats sacrificed 4 hours after the last injection of ACE. In the 24 hour-fasted group the liver glycogen level of the ACE

TABLE 1. EFFECTS OF ADRENAL CORTICAL EXTRACT ON ADIPOSE TISSUE AND LIVER GLYCOGEN AND BLOOD SUGAR LEVELS OF RATS

Experiment	No. rats	Body wt., grams	Adipose tissue glycogen, gms./100 gm. fat	P	Liver glycogen, gms./100 gm.	P	Blood sugar, mg./100 ml.	P
8 ml. A.C.E.* 24 hr. fast	8	95	0.067±0.016	0.2	1.88 ±0.140	< .01	115± 8.9	>0.1
8 ml. Saline 24 hr. fast	9	95	0.094±0.025		0.667±0.117		100± 3.9	
8 ml. A.C.E.* 48 hr. fast	4	93	0.062±0.006	>0.05	1.62 ±0.090	>0.1	110± 9.9	>0.1
8 ml. Saline 48 hr. fast	4	97	0.100±0.018		1.31 ±0.247		112± 3.6	
8 ml. A.C.E.* 1 gm. Glucose† 48 hr. fast	4	96	1.714±0.300	>0.5	3.22 ±0.159	<.05	175±11.7	>0.5
8 ml. Saline 1 gm. Glucose† 48 hr. fast	4	84	1.491±0.281		2.21 ±0.370		163±40.4	
1 ml. Lipoadrenal‡‡ Cortex 48 hr. fast	9	93	0.102±0.015	>0.5	1.29 ±0.179	< .01	125± 4.2	<.01
1 ml. Peanut Oil‡ 48 hr. fast	10	95	0.091±0.008		0.503±0.113		105± 5.2	
1 ml. Lipoadrenal Cortex & 1 gm. Glucose/100 gm.§ 48 hr. fast	10	143	0.443±0.046	>0.5	4.64 ±0.172	<.01	228±18.4	0.1
1 ml. Peanut Oil‡ & 1 gm. Glucose/100 gm.§ 48 hr. fast	10	140	0.455±0.026		3.59 ±0.236		219±11.1	

* 1 ml. of Upjohn's Adrenal Cortex Extract with alcohol evaporated off under vacuo injected subcutaneously hourly for 8 hours in rats sacrificed 4 hours after last injection.
† 2 ml. of 50% glucose given by stomach tube at 8th hour.
‡ Injected subcutaneously 8 hours before rats were sacrificed.
§ 1 ml. of 50% glucose per 100 gms. body weight given by stomach tube 2 and 5 hours after injection of lipoadrenal cortex or peanut oil.

treated series was significantly higher than in the saline-treated controls. In the small 48 hour-fasted series there was no difference in liver glycogen concentrations. However, one value in the control group was unusually high for a 24 hour-fasted rat, which probably accounts for the failure to demonstrate a significant difference in this small series. In the third set of experiments rats similarly treated with ACE and saline were given in addition 2 ml. of 50% glucose by stomach tube 4 hours before sacrifice. Again there were no differences in adipose tissue glycogen or blood sugar, but the liver glycogen levels of the treated group were higher. Likewise, negative results followed treatment with Lipoadrenal Cortex when adipose

tissue glycogen concentrations were determined in 48 hour-fasted rats or similarly fasted rats given 1 ml. of 50% glucose 6 and 3 hours before sacrifice. The Lipoadrenal Cortex was administered as a single subcutaneous injection of 1 ml. 8 hours before sacrifice. The controls received an equal volume of peanut oil. The liver glycogen levels of the LAC treated animals were significantly elevated in both the fasted and fed groups, as was the blood sugar in the fasted series.

The changes in dorsal adipose tissue glycogen were also insignificant when ACTH (Sayers) was administered (Table 2). This was true in both the 48 hour-fasted rats given 1 mg. of ACTH intraperitoneally 8 and 5 hours before sacrifice and in those given 1.5 mg. ACTH at these times as well as 2 ml. of 50% glucose 5 and 3 hours before sacrifice. In neither of these cases was there any change in the liver glycogen. Since it seemed possible that too short a time had been allowed for the effects of adrenal stimulation to become felt, the experiment was repeated in 48 and 24 hour fasted rats given 2 mg. of ACTH (Armour) 24, 18 and 12 hours before sampling, a total dose of 6 mg. In both experiments significant increases in adrenal weight occurred in the ACTH treated animals, but no changes in adipose tissue glycogen or blood sugar. A small increase in liver glycogen occurred in the 48 hour-fasted ACTH treated animals but not in the 24 hour-fasted group. There is no ready explanation for the failure to note greater increases in liver glycogen unless it be related to the relatively high posterior pituitary contamination of this particular lot of ACTH (0.13 unit oxytocin and 0.26 unit pressor per milligram). However, in subsequent experiments in which a lot of ACTH with rather lower posterior pituitary contamination was used, equally unimpressive effects on liver glycogen were noted (see below).

From these data, then, there is no evidence for a role of the adrenal cortex in glycogen synthesis in the dorsal interscapular fat body of the rat.

Effects of Adrenalectomy on Fat Glycogen Stores

In Table 3 are seen the adipose tissue and liver glycogen and blood sugar levels of 24 hour-fasted and glucose fed intact and adrenalectomized rats. The latter were prepared 5 days before the study and maintained on 0.5 mg. of desoxycorticosterone acetate daily and saline drinking water. The fed group received 500 mg. glucose per 100 grams body weight as 50% glucose by stomach tube 8 hours before sacrifice. As anticipated, the blood sugar and liver glycogen concentrations of the adrenalectomized rats were lower than their corresponding controls. On the other hand, the adipose tissue glycogen of the fasted adrenalectomized rats was not significantly different from the controls, but that of the glucose fed adrenalectomized animals was significantly lower than the controls and not significantly

TABLE 2. EFFECT OF ADRENOCORTICOTROPHIC HORMONE ON ADIPOSE TISSUE AND LIVER GLYCOGEN AND BLOOD SUGAR LEVELS

Experiment	No. rats	Mean body wt., grams	Adr. wt., mg./100 gms.	P	Adipose tissue glycogen, gms./100 gm. fat	P	Liver glycogen, gms./100 gm. liver	P	Blood sugar, mg./100 ml.	P
48 Hour Fast										
2 mg. ACTH*†	14	105	—	—	0.057 ± 0.012	>0.5	0.680 ± 0.106	>0.5	112 ± 12.7	<0.2
Control†	20	114	—	—	0.052 ± 0.007		0.728 ± 0.077		92 ± 3.0	
3 mg. ACTH‡ 2 gm. Glucose§ p.o.	14	103	—	—	0.766 ± 0.125	>0.5	3.49 ± 0.162	>0.5	368 ± 60.4	< .05
Control 2 gm. Glucose§ p.o.	22	113	—	—	0.690 ± 0.106		3.10 ± 0.307		229 ± 8.4	
6 mg. ACTH‖	8	96	39.2 ± 1.58	< .01	0.109 ± 0.011	>0.1	0.306 ± 0.095	< .01	85 ± 4.9	> 0.5
Control	7	104	27.9 ± 2.21		0.160 ± 0.025		0.046 ± 0.023		84 ± 4.1	
24 Hour Fast										
6 mg. ACTH‖	19	99	37.5 ± 2.70	< .01	0.127 ± 0.009	>0.1	0.208 ± 0.065	>0.1	118 ± 5.5	>0.1
Control	18	116	28.9 ± 0.32		0.155 ± 0.015		0.338 ± 0.099		128 ± 6.4	

* 1 mg. of ACTH injected intraperitoneally at 0 and 3 hours. Sacrificed 8 hours after first injection.
† 2 ml. of normal saline by stomach tube at 3 and 5 hours.
‡ 1.5 mg. of ACTH injected intraperitoneally at 0 and 3 hours. Sacrificed 8 hours after injection.
§ 2 ml. of 50% glucose by stomach tube at 3 and 5 hours.
‖ 2 mg. ACTH injected intraperitoneally at 0, 6 and 12 hours, rats sacrificed 24 hours after first injection.

different from the fasted adrenalectomized rats. This confirms the earlier report of Tuerkischer and Wertheimer (1942) that the adrenalectomized rat allowed to eat a high carbohydrate died ad lib after a fast does not accumulate adipose tissue glycogen to the same degree as normal. Although the factor of food intake was controlled in our experiments, the significance of altered rates of absorption in the adrenalectomized rats influencing the observed results cannot be determined with certainty. In the intact animal maximal adipose tissue glycogen deposition after a single dose of glucose by mouth occurs in 4 hours (Engel and Scott, 1950), the level thereafter

TABLE 3. EFFECT OF ADRENALECTOMY ON ADIPOSE TISSUE AND LIVER GLYCOGEN AND BLOOD SUGAR LEVELS OF 24 HOUR FASTED RATS

Experiment	No. rats	Body weight, grams	Adipose tissue glycogen, gms./100 gm. fat	P	Liver glycogen, gms./100 gm. liver	P	Blood sugar, mg./100 ml.	P
Adrenalectomized*	27	198	0.155±0.011	.5	0.044±0.002	.01	87±2.1	.01
Control	27	180	0.139±0.016		0.238±0.031		101±2.4	
Adrenalectomized* +500 mg. Glucose/100 gm. p.o.†	17	134	0.212±0.030	<.01	1.39 ±0.198	<.01	87±4.0	<.01
Control+500 mg. Glucose/100 gm. p.o.†	18	131	0.357±0.031		2.22 ±0.228		107±2.8	

* Adrenalectomized 5 days before the experiment and maintained on 0.5 mg. of desoxycorticosterone acetate daily by injection and saline drinking water.
† 1.0 ml. of 50% glucose per 100 grams body weight given by stomach tube 8 hours before rats were sacrificed.

declining. One might anticipate that if adipose tissue glycogen deposition after glucose were normal in the adrenalectomized rat, the effect of delayed absorption would be to move the peak of glycogen accumulation to a later time and hence that the level of glycogen in the adrenalectomized rats would be at least as great if not greater than normal at 8 hours when glucose absorption should be complete. This interpretation suggests that the lower fat glycogen levels in the glucose fed adrenalectomized rats represent a true defect in glycogen formation in this preparation.

Interrelation of Insulin and Adrenal Cortex in Fat Glycogen Synthesis

No positive effects of adrenal hormone in fat glycogen accumulation having been demonstrated, it was considered possible that since insulin markedly elevates adipose tissue glycogen (Wertheimer and Shapiro, 1948, Renold *et al.*, 1950, Engel and Scott, 1950) and since in general the adrenal cortex exerts an antagonistic effect to insulin in intermediary metabolism, an action of the adrenal on adipose tissue glycogen might be demonstrated in the insulin treated rat. It was anticipated that if any effect of the adrenal were to be demonstrated it would be an inhibitory one on the insulin response in adipose tissue glycogen. The opposite proved

to be the case in rats treated with cortisone or ACTH and insulin; no such effect was noted in rats treated with lipoadrenal cortex and insulin.

In all the following experiments with intact rats (Table 4) the design was identical except for the administration of lipoadrenal cortex (LAC), cortisone acetate or ACTH to 2 groups of rats in each of the three different experiments, the remaining two groups of rats being treated identically in all the experiments. In each experiment, except that with LAC, 20–28 rats weighing 150–200 grams were divided into 4 equal groups and fasted 48 hours. All groups were given 1 ml. of 50% glucose per 100 grams

TABLE 4. COMBINED EFFECTS OF GLUCOSE, INSULIN, AND LIPOADRENAL CORTEX, CORTISONE ACETATE OR ACTH ON ADIPOSE TISSUE AND LIVER GLYCOGEN AND BLOOD SUGAR OF 48 HOUR FASTED RATS

Group	Experiment	Adipose tissue glycogen, gms./100 gm. fat	Liver glycogen, gms./100 gm. liver	Blood sugar, mg./100 ml.
1	Glucose	0.464±0.039 (43)	4.00±0.140 (44)	155± 5.2 (45)
2	Glucose+Insulin	0.819±0.071 (32)	2.63±0.128 (34)	129± 5.4 (34)
3	Glucose+LAC*	0.443±0.046 (10)	4.64±0.172 (10)	228±18.4 (10)
4	Glucose+LAC*+Insulin	0.671±0.036 (32)	2.76±0.089 (34)	157± 8.5 (34)
1	Glucose	0.425±0.029 (21)	4.14±0.300 (10)	125± 4.9 (10)
2	Glucose+Insulin	0.708±0.045 (37)	1.86±0.105 (10)	97± 7.1 (10)
3	Glucose+Cortisone†	0.532±0.045 (15)	5.44±0.587 (10)	146±21.6 (10)
4	Glucose+Cortisone†+Insulin	0.911±0.043 (24)	3.39±0.419 (10)	99± 4.3 (10)
1	Glucose	0.397±0.036 (25)	3.85±0.119 (26)	141± 2.7 (26)
2	Glucose+Insulin	0.597±0.053 (30)	1.88±0.103 (29)	106± 4.9 (30)
3	Glucose+2.5 mg. ACTH‡	0.574±0.065 (13)	3.95±0.192 (13)	139± 5.1 (13)
4	Glucose+2.5 mg. ACTH‡+Insulin	0.756±0.062 (13)	2.56±0.172 (13)	125±11.5 (13)
3	Glucose+7.5 mg. ACTH§	0.449±0.042 (12)	3.84±0.150 (12)	138± 4.4 (12)
4	Glucose+7.5 mg. ACTH§+Insulin	0.801±0.069 (11)	2.10±0.246 (12)	91± 5.7 (12)

All rats received 1 ml. of 50 per cent glucose by stomach tube 3 and 6 hours before sacrifice.
Insulin-treated rats received 2 units of insulin intraperitoneally 6 hours before sacrifice.
* Lipoadrenal cortex, 10 glycogenic units 8 hours before sacrifice.
† Cortisone Acetate, 2.5 mg. 24 and 48 hours before sacrifice.
‡ ACTH, 2.5 mg. in divided doses over 24 hours.
§ ACTH, 7.5 mg. in divided doses over 48 hours.

by stomach tube 6 and 3 hours before sacrifice. Group 1 had no further therapy; Group 2 got 2 units of insulin intraperitoneally 6 hours before sacrifice; Group 3 got either ACTH, LAC or cortisone in the dosage and at the time recorded below; and Group 4 got 2 units of insulin 6 hours before sampling in addition to either ACTH, LAC or cortisone exactly as in Group 3. Although the results were generally quite uniform in each group on any day, unexplained variations in the magnitude of insulin response took place from day to day. For this reason each experiment consisting of the four groups of animals was repeated 2–4 times in order to establish the statistical validity of the results. We suspect that fluctuations in room temperature, seasonal factors and variations in endogenous adrenal cortical secretion may have been concerned with the more variable response of the insulin treated groups. The results of these experiments are recorded in Table 4. Note firstly that adipose tissue and liver glycogen levels and blood sugars of the glucose treated group in the three experiments compare very closely. In the insulin groups, the adipose tissue glyco-

gen levels are in all cases significantly higher than the glucose groups, but range from 0.597 ± 0.053 mg. to 0.819 ± 0.071 mg. per 100 gm. fat in the three groups, the latter being significantly higher than the former. Liver glycogen and blood sugar levels after insulin were in all cases significantly lower than their controls. In the first experiment either 1 ml. of Lipoadrenal Cortex containing 10 glycogenic units per milliliter or 1.5 ml. of pooled hog adrenal extract in oil containing 7 glycogenic units per milliliter were given subcutaneously 2 hours before the first dose of glucose or glucose and insulin. The glucose and LAC group in this experiment was studied at a different time and is the same as recorded in Table 1. In the group receiving LAC, insulin and glucose the adipose tissue glycogen was slightly lower than the insulin-glucose treated group, but not significantly so. The liver glycogen values of both insulin treated groups were identical and significantly lower than either the glucose or glucose and LAC groups. LAC elevated the blood sugar in both the insulin and the glucose groups compared to their controls.

A different result with respect to adipose tissue glycogen is seen in the cortisone series. Here a single subcutaneous injection of 2.5 mg. of cortisone acetate as a crystalline suspension in saline was given at the beginning of the fast and again 24 hours before the rats were sacrificed. A small but statistically insignificant increase in adipose tissue glycogen was noted in the cortisone and glucose group, while a striking and highly significant increase in fat glycogen occurred in the cortisone-glucose-insulin group. This level was substantially higher than all other groups. The liver glycogen levels of the cortisone group were highest but because of considerable variation in the group were not significantly higher than the controls. On the other hand, cortisone significantly decreased the fall in glycogen seen after insulin, but not the blood sugar.

The ACTH experiment (Table 4) was carried out at 2 different dose levels of the hormone. In one 2.5 mg. of ACTH were given in divided doses during the 24 hours prior to sacrifice while in the other 7.5 mg. were given over 48 hours. With the smaller dose, a small, but significant increase in adipose tissue glycogen took place, the only experiment in this entire study where such an effect was noted. Both doses of ACTH, when given in conjunction with insulin and glucose, induced significant increases in adipose tissue glycogen compared to all other treatments. In this experiment the effect of insulin and glucose alone on adipose tissue glycogen was the smallest and the values achieved after ACTH and insulin and glucose were not greater than seen after insulin and glucose alone in the two previous experiments. The latter were performed in the summer and fall, while the ACTH experiment was conducted in early spring. No significant effects on liver glycogen or blood sugar were induced by ACTH, either in

the control or insulin-treated rats. The metabolic activity of this particular lot of ACTH (Armour) is attested to by its production of glycosuria in 2 patients with rheumatoid arthritis at dose levels of 35 to 50 mg. per day.

One possible explanation for the higher adipose tissue glycogen levels

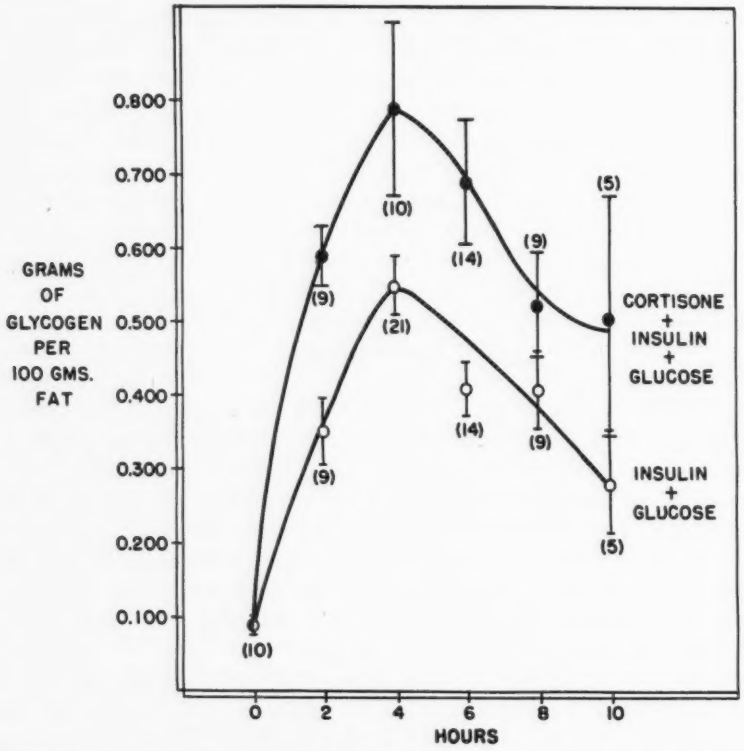

FIG. 1. Changes in the glycogen content of the dorsal interscapular fat of 48-hour-fasted rats treated with cortisone, insulin and glucose and with insulin and glucose. The numbers in parentheses refer to the number of animals in each group.

after adrenal hormone plus insulin and glucose might be that the adrenal cortex alters the time at which maximal glycogen accumulation is achieved, although the total glycogen synthesis in the two cases might be the same. That this is not so is seen in Figure 1. In this experiment one group of rats was given 5 mg. of cortisone acetate in divided doses at 48 and 24 hours before administration of glucose and insulin and another group left untreated until this time. At the end of the 48 hour fast all rats were given

1 unit of insulin per 100 grams body weight intraperitoneally and 1 ml. of 50 per cent glucose per 100 grams by stomach tube. The rats in both groups were sacrificed at two hour intervals up to 10 hours after glucose and insulin. As seen in Figure 1, maximal glycogen levels were reached at 4 hours in both groups and the glycogen values of the cortisone treated animals were always higher. The insulin curve recorded here is the same one as previously reported (Engel and Scott, 1950) but was determined concurrently with the cortisone and insulin curve.

TABLE 5. EFFECT OF INSULIN AND GLUCOSE ON THE ADIPOSE TISSUE AND LIVER GLYCOGEN AND BLOOD SUGAR LEVELS OF ADRENALECTOMIZED RATS FASTED 24 HOURS

Experiment	No. rats	Adipose tissue glycogen, gms./100 gm. fat	P	Liver glycogen, gms./100 gm. liver	P	Blood sugar, mgm./100 ml.	P
Adrenalectomized 400 mg. Glucose/100 gm. subcutaneously 1 unit Insulin, i.p.*	7	0.274±0.034	<.01	0.024±0.002	<.01	34± 7.0	<.01
Control-Glucose+Insulin*	10	0.470±0.070		1.24 ±0.179		143± 9.1	
Adrenalectomized 250 mg. Glucose i.v. 0.4 unit Insulin, subcutaneously†	8	0.207±0.028	<.01	1.22 ±0.076	>.1	76±12.0	>.05
Control Glucose and Insulin	7	0.352±0.032		1.30 ±0.062		106± 7.5	

* 24 hour fast. 1 ml. 20% glucose/100 gm. subcutaneously 2 and 4 hours, 1 unit of insulin intraperitoneally 4 hour before sacrifice.
† 48 hour fast. 0.5 ml. 50% glucose/100 gm. intravenously 4 hours, 0.2 unit insulin subcutaneously 2 and 4 hours before sacrifice.

Additional observations were made to determine whether the adrenalectomized rat given insulin and glucose had a normal or lesser ability to form adipose tissue glycogen than the intact rat similarly treated with insulin and glucose. This experiment was made difficult by the high sensitivity of the adrenalectomized rats to doses of insulin which induce adipose tissue glycogen increases and the uncertainty of absorption of glucose in the adrenalectomized animals. Two experiments were done (Table 5). In the first glucose was given subcutaneously as 1 ml. per 100 grams of a 20 per cent solution to 24 hour fasted rats 4 and 2 hours before sacrifice and 1 unit of insulin intraperitoneally 4 hours before sampling. In this experiment the adipose tissue and liver glycogen and blood sugar levels of the adrenalectomized rats were all significantly lower than the controls. Since only 7 of the 20 adrenalectomized rats survived this procedure and there was uncertainty as to whether the glucose was absorbed from the subcutaneous depot in this group a second experiment was designed to avoid some of these difficulties. In this experiment, 15 rats were adrenalectomized 5 days before a 48 hour fast was begun. During this period they were maintained on Purina chow, 5 per cent glucose and saline in their drinking bottles as well as 3 ml. of this same solution intraperi-

toneally, 0.5 mg. desoxycorticosterone acetate and 0.3 ml. of adrenal cortical extract subcutaneously daily. At the beginning of the 48 hour fast saline alone was put in the drinking bottles and only desoxycorticosterone given by injection. At the end of the 48 hour fast all rats were given 0.5 ml. of 50 per cent glucose per 100 grams intravenously under light nembutal anesthesia and 0.2 unit insulin subcutaneously. Two hours later each rat was given an additional 0.2 unit insulin and all were sacrificed at 4 hours. Eight adrenalectomized rats survived this procedure. As seen in Table 5, the adipose tissue glycogen and blood sugar levels of the insulin treated rats were again significantly lower than the controls, while the liver glycogen values were the same. Since the problem of glucose absorption was eliminated in this experiment it would appear that the adrenalectomized rat has a lesser ability to form glycogen in the dorsal interscapular fat body after insulin and glucose than does the normal animal.

DISCUSSION

It is apparent from these experiments that the adrenal cortex by itself does not seem to have any direct positive influence on net glycogen synthesis in the dorsal interscapular fat body of the rat, at least under the experimental conditions herein described. Baker *et al.* (1950) have also been unable to detect an effect of ACTH on the glycogen content of brown fat of rats, measured histochemically. A variety of different potent adrenal and pituitary preparations failed to influence adipose tissue glycogen in the fed or fasted state. The adrenalectomized animal likewise showed normal adipose tissue glycogen levels when fasting, but, on the other hand, failed to increase glycogen levels after glucose feeding to the same degree as did the normals. This finding confirms that of Tuerkischer and Wertheimer (1942). The lack of effect of adrenal hormone alone contrasts with the situation when insulin is given to the ACTH or cortisone treated rats. Under these circumstances a striking accentuation of the usual stimulating effect of insulin on adipose tissue glycogen accumulation occurs. There is no immediate explanation as to why a similar increase did not take place in the rats treated with lipoadrenal cortex and insulin. It may be significant that these animals were under adrenal hormone therapy for only 8 hours compared to 24–48 hours in the ACTH and cortisone series. The data on the effect of insulin in adrenalectomized rats suggest that in the absence of the adrenal cortex maximal insulin effects are not achieved. However, since there were no adrenalectomized rats given glucose alone as controls, it cannot be concluded that insulin does not stimulate adipose tissue glycogen synthesis in the adrenalectomized animal.

The apparent synergism between the adrenal cortex and insulin in adi-

pose tissue glycogen accumulation may have some bearing on certain hitherto unexplained variations in adipose tissue glycogen. It was noted above that whereas the adipose tissue glycogen concentrations after glucose are extraordinarily constant and reproducible, the effects of insulin are variable. Might this be related to spontaneous variations in levels of endogenous adrenal cortical hormone secretion? Renold *et al.* (1950) have reported that the same dose of insulin and glucose induced a much greater increase in adipose tissue glycogen in alloxan diabetic animals than in normals. The alloxan diabetic rat usually has hypertrophic adrenals and there is evidence that the untreated diabetic may have increased adrenal cortical activity. When rats are exposed to the cold and then given insulin and glucose, very large increases in adipose tissue glycogen take place (Engel, 1950). Studies are in progress on the effects of varying doses of cortisone on the adipose glycogen response to insulin and glucose in the adrenalectomized rat. It would be premature at this time to attempt to interpret the observed changes in adipose tissue glycogen deposition following insulin and cortisone or ACTH therapy in terms of specific steps in the metabolism of carbohydrate and fat. If it be assumed with Wertheimer and Shapiro (1948) that the increased glycogen accumulation after insulin parallels or represents increased conversion of carbohydrate to fat, the ability of the adrenal cortex to further increase the glycogen concentration could be due either to a stimulation of glycogen synthesis or to an inhibition of glycogen breakdown. In the former case the adrenal effect might be interpreted as a stimulating one on liponeogenesis and in the latter case as an inhibitory one. Until more information of a fundamental nature is obtained concerning the synthesis, degradation and precise metabolic role of glycogen in adipose tissue, no conclusions can be reached. Currently available data on other changes in adipose tissue under hormone treatment do not help resolve this problem. Fawcett and Jones (1949) have reported that ACTH failed to increase the neutral fat content of the interscapular adipose tissue of the mice, while Baker, Ingle and Li (1950), using histochemical techniques, describe an increase in the glyceride content of brown fat of rats treated with ACTH. This could be due either to an increase in the total amount of fat or in the proportion of short-chain fatty acids in the fat. Both groups of investigators described a depletion of the fat of the interscapular fat body after hypophysectomy. Fawcett and Jones (1949) were able to prevent this depletion with ACTH while Baker *et al.* (1950) could not. Stoerk and Porter (1950) have recently reported that cortisone will prevent the loss of adipose tissue that occurs in the adrenalectomized rat during food restriction. Selye and Timiras (1949), on the other hand, report fat depletion in the dorsal fa body after

stress and imply that this is part of the alarm reaction mediated by the adrenal-pituitary system. In none of our studies was any significant change in the total weight of the dorsal fat body noted as a result of the experimental procedures. Regrettably, the weight of the entire fat body was not measured in the adrenalectomized rats. It is clear that the available data are not yet sufficiently consistent or extensive to allow for the interpretation of our data in terms of a specific effect of the adrenal cortex on fat synthesis.

SUMMARY

Administration of adrenal cortical extract, Lipoadrenal Cortex, cortisone or ACTH had no effect on the concentration of glycogen in the dorsal interscapular fat pad of 24 or 48 hour fasted male rats or similarly fasted rats given large amounts of glucose by stomach tube. The expected changes in liver glycogen and blood sugar occurred in most, but not all experiments.

The adipose tissue glycogen concentration of 24 hour fasted NaCl and desoxycorticosterone acetate maintained adrenalectomized rats was the same as that of untreated intact control rats. The adipose tissue glycogen concentration after oral glucose administration was significantly lower in adrenalectomized rats than in their controls given glucose, and not significantly different from that in intact or adrenalectomized rats not given glucose. Liver glycogen and blood sugar levels were lower in the adrenalectomized rats.

The increase in adipose tissue glycogen generally seen in the 48 hour fasted rat given insulin and glucose was significantly accentuated by pretreatment of the rats with either ACTH or cortisone acetate for 24 or 48 hours but not by Lipoadrenal Cortex for 8 hours. The fall in liver glycogen and blood sugar after insulin was not consistently prevented by ACTH, cortisone or Lipoadrenal Cortex treatment. When measured at 2 hour intervals up to 10 hours the adipose tissue glycogen levels of cortisone treated, 48 hour fasted rats given a single dose of glucose and insulin were consistently higher than controls given glucose and insulin.

Adrenalectomized rats given glucose subcutaneously or intravenously had significantly lower adipose tissue glycogen levels after insulin than did similarly treated intact rats. In the groups receiving glucose intravenously there was no significant difference in the liver glycogen level between the control and adrenalectomized rats.

The significance of the adrenal-insulin relationship in adipose tissue glycogen synthesis is discussed.

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METABOLIC EFFECTS OF SOME HALOGENATED ACRYLIC ACID ANALOGUES OF THYROXINE[1]

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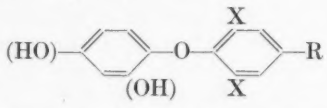
IN THE literature of the study of the portions of the thyroxine molecule structure which are required for physiological activity, three general points of attack are prominent: (1) the specific type as well as number of atoms of halogen, (2) the diphenyl ether skeleton, (3) the alanine side-chain. The work has recently been summarized by Frieden and Winzler (1948), who concluded that thyroxine-like activity was dependent upon the following combination of features:



with R a wide variety of active groups. Because of the recent report by Harington (1948) concerning activity shown by the thio-ether analogue, this modification should also be included.

It is the purpose of this paper to report work bearing principally upon the number and type of halogens and influence of the R side-chain.

METHODS

The animals used were young adult male albino rats of the Sprague-Dawley strain, 300–350 grams in weight. They were placed on the special feeding regime used in this laboratory for producing the post-absorptive condition (Barker, 1949). Oxygen consumption studies were carried out on trained animals in a four-chamber Benedict closed-circuit apparatus. Following equilibration for 20–30 minutes, the metabolic rate was measured for two or three 30-minute periods.

The compounds studied were synthesized as reported by Wawzonek and Wang (1950). In each case, the sodium salt was made up in water solution to be isotonic with 0.9% NaCl. In most instances, propylene glycol was required in varying amounts to effect good solution. Injections were made subcutaneously with a small needle, the sites being rotated to avoid undue damage and necrosis.

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RESULTS AND DISCUSSION

Table 1 shows the results obtained on both normal and thyroidectomized rats with the 8 compounds employed. For convenience in comparison, the doses injected into normal animals are calculated as molar equivalents of the 61 μgm. of DL-thyroxine[2]/kg. body wt./day required to produce a 161 cc. O_2/kg./hr. average increase on the fourth, fifth and sixth days of

TABLE 1. INCREASES IN OXYGEN CONSUMPTION OF NORMAL AND THYROIDECTOMIZED RATS PRODUCED BY THYROXINE ANALOGUES

Compound	Normal rats			Thyroidectomized rats		
	Dose	BMR Incr.	% thyroxine	Dose	Incr.	% thyroxine
		cc. O_2/kg./hr.				
DL-Thyroxine	61	161	100	12	227	100
"MDBA": 4-(4′-methoxyphenoxy)-3,5-dibromoaniline						
	10,000×	8	0.001	500×	175	0.15
"MDIA": 4-(4′-methoxyphenoxy)-3,5-diiodoaniline						
	5,000×	224	0.027	100×	102	0.45
"MDBC": 4-(4′-methoxyphenoxy)-3,5-dibromophenylacrylic acid						
	2,500×	49	0.008	1,000×	55	0.024
"MTBC": 4-(4′-methoxy-3′,5′-dibromophenoxy)-3,5-dibromophenylacrylic acid						
	1,000×	97	0.060	100×	138	0.61
"MDIC": 4-(4′-methoxyphenoxy)-3,5-diiodophenylacrylic acid						
	500×	28	0.02			
	2,000×	304	0.06	1,000×	91	0.04
"HDIC": 4-(4′-hydroxyphenoxy)-3,5-diiodophenylacrylic acid						
	1,000×	338	0.21	50×	43	0.38
				250×	83	0.15
"HTIC": 4-(4′-hydroxy-3′,5′-diiodophenoxy)-3,5-diiodophenylacrylic acid						
	100×	213	1.32	50×	333	2.93
"HDIP": 4-(4′-hydroxyphenoxy)-3,5-diiodophenylpropionic acid						
	100×	123	0.77	50×	217	1.91
	500×	870	1.09			

injection. In the case of the thyroidectomized, highly consistent results were obtained with 12 μgm. of DL-thyroxine/kg./day, amounting to an increase of 227 cc. O_2/kg./hr. after 10 days of injections. It should be emphasized that the (quantitative) evaluation of the effectiveness of any thyroxine analogue in terms of thyroxine itself depends upon the dose of the hormone used as reference. In each instance, computation was as follows:

$$\text{Percentage of thyroxine activity} = \frac{\text{increased } O_2 \text{ consumption with compound}}{\text{increased } O_2 \text{ consumption with thyroxine}} \times \frac{1}{\text{molar equivalents of compound used}} \times 100.$$

Thus, if a dose 500 times equimolar with 12 μgm. of thyroxine produced

[2] DL-thyroxine was generously furnished by Dr. K. W. Thompson of Organon, Inc., Orange, N. J.

an identical rise in BMR, the material is said to have 0.2% thyroxine activity.

The 4-(4′-methoxyphenoxy)-3,5-dibromo aniline gave 0.15% of thyroxine response in the thyroidectomized animals, but even a dose 10,000 times the standard 61 μgm. of thyroxine gave such a slight response in the normal rats that it has merely been listed as less than 0.001%. When iodine was substituted for bromine in the above aniline analogue, activity rose to 0.45% in the thyroidectomized animals, and 0.03% in the normals.

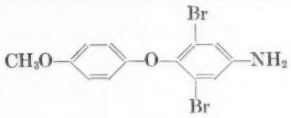
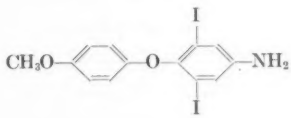
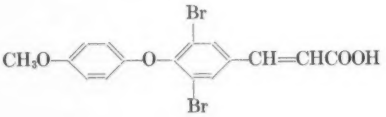
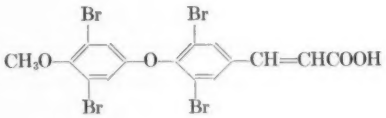
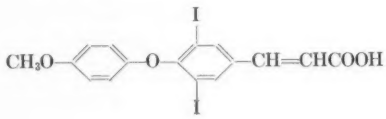
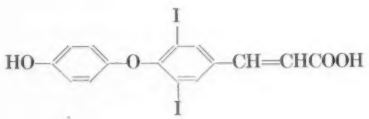
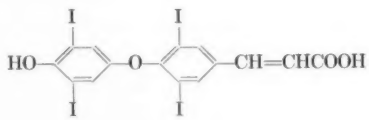
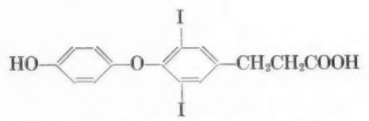
The next 3 compounds shown are all halogenated 4-(4′-methoxyphenoxy)-phenylacrylic acids; with bromine in the 3 and 5 positions a very slight amount of stimulation of metabolism was shown, 0.01% in the normals and twice that in the athyroid rats. When 2 more bromines were added, in the 3′ and 5′ positions, activity increased 6-fold in the normals and 30-fold in the thyroidectomized. With iodine in the 3 and 5 positions, metabolism was stimulated about the same amount in both groups of animals and to a somewhat greater extent than by the corresponding dibromo compound.

The next compound tested, 4-(4′-hydroxyphenoxy)-3,5-diiodophenylacrylic acid, exhibited an activity about seven times increased over that of the 4′-methoxy analogue strongly suggesting that the methyl ether must be split before the latter type of compound can exert an effect on metabolism. This is in accord with the results reported by Myers (1932) that 0-methyl thyroxine was much less effective than free thyroxine when tested on amphibian metamorphosis and was inactive in the human. In contrast, Loeser *et al.* (1938) claimed increased effectiveness from the 0-methyl ether administered by mouth.

The most active compound of all was the 4-(4′-hydroxy-3′,5′-diiodophenoxy)-3,5-diiodophenylacrylic acid shown next in the table, the 2 additional iodines enhancing the effect on normals six times and on thyroidectomized eleven times. Even such increases, however, produced final activities only 1.3 and 2.9% as great as the corresponding amounts of thyroxine in the normals and athyroid, respectively.

The final compound, 4-(4′-hydroxyphenoxy)-3,5-diiodophenylpropionic acid, was 4 to 7 times as active in the corresponding two groups of animals as was the unsaturated acrylic acid analogue. Although the unsaturated acid side-chain might be aminated to the amino acid form, it is highly doubtful if the saturated chain would so be changed. This finding of greater activity with the saturated side-chain strongly suggests that the presence of the amphoteric amino acid is not essential. If this type of compound does enter into chemical combination with protein material, it would of necessity become a terminal group, since it could form only one peptide bond. It is not entirely impossible that the alanine side-chain is of value

TABLE 2. SUMMARY OF THYROXINE ANALOGUE EFFECTS

Compound	% DL-thyroxine activity		
	Normal rats	Thyroidect. rats	Tadpoles
	<0.001	0.15	
	0.03	0.45	
	0.01	0.02	4
	0.06	0.61	
	0.04	0.04	10
	0.21	0.27	24
	1.32	2.93	290
	0.93	1.91	

only during the stages of formation of thyroxine from tyrosine in the thyroid gland.

Although these results show that bromine in the 3,5 and 3′,5′ positions does impart some metabolism-stimulating activity to the fundamental diphenyl ether structure, they also confirm much earlier work (cf. Abderhalden and Wertheimer, 1928; Gaddum, 1929; Lerman and Harington, 1949) that bromine is less effective than iodine. Similar results have been reported for chlorine (Lerman and Harington, 1949) and fluorine (Cortell, 1949).

From Table 2, it can be seen that the four members of the present series of compounds which were tested by Frieden and Winzler (unpublished) revealed activities in amphibian metamorphosis 100 to 400 times as great as those exerted on the metabolic rates of our rats. It is interesting that a similar difference in order of sensitivity is obtained in comparing 4-benzyloxy-3,5-diiodobenzoic acid as an inhibitor of thyroxine stimulation of metabolism (Frieden and Winzler, 1949; Maclagen *et al.*, 1949; Barker *et al.*, 1950).

SUMMARY

The results of these experiments support the concept of a lack of specific dependence upon the side chain for the metabolic activity of thyroxine.

Setting up a methyl ether on the terminal hydroxy group of the outer phenyl ring decreased the thyroxine-like action of one of a diiodo unsaturated analogue.

Iodine in the 3,5 configuration has again been found to impart more activity than bromine and addition of two more atoms of the particular halogen in question in the 3′ and 5′, affects the potency favorably.

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THE ENERGY METABOLISM OF NORMAL RABBITS AS INFLUENCED BY THYROTROPIC HORMONE, INSULIN, THYROXIN, AND THE COMBINATION OF INSULIN AND THYROXIN*

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IN PREPARATION for a study of the influence of several hormones on the growth and metastasis of the Brown-Pearce epithelioma of the rabbit, it was considered desirable to ascertain the effects of certain of these hormones singly and in combination, on the respiratory metabolism of normal rabbits. The data recorded in this paper were obtained on New Zealand white males in the years 1940 and 1941. Work on the B-P tumor was interrupted by World War II in 1942 and was resumed by permission of Professor Fenn in 1946. Results on the respiratory metabolism of the tumor bearing rabbit will be reported later.

A quite complete review of the energy metabolism of rabbits is contained in the monograph by F. G. Benedict (1938), entitled "Vital Energetics: A Study of Comparative Basal Metabolism." Since that year several other papers have appeared contributing observations pertinent to the present discussion. R. C. Lee (1939) from the same laboratory reported an extensive research into the factors influencing heat production in the rabbit and suggesting certain standard conditions to be observed when measuring its basal rate.

The effect of insulin and thyroxin on the energy metabolism of rabbits has not received much attention, though other species have been extensively investigated. Hawley and Murlin (1925) were among the first to explore the effects of insulin not merely on the blood sugar of this animal, which was at that time the favorite test subject for the standardization of insulin, but on the whole respiratory metabolism. The object was to see whether or not the total energy metabolism could also be used for standardization purposes. The project was however somewhat premature, for the reason that insulin preparations were not then sufficiently purified to give constant results. However, the results obtained are essentially in

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accord with the results of the present work performed 15 years later (1940) and making use of more highly refined preparations.

The influence of thyroxin on the rabbit was investigated by Le Breton and Schaeffer (1935) who gave 1.5 to 2.5 mg. per kg. body weight and obtained increased heat production of 60% on the 2nd day. Mahaux (1938, a and b) published two papers containing extensive data obtained from the administration of thyroxin and thyrotropic hormone, both singly and in combination, to normal adult rabbits. The results of these studies are expressed not in the more recently accepted manner of calories per square meter per hour, but in cubic centimeters of oxygen per minute. The percentage increases in oxygen uptake, however, make possible certain comparisons with our own results, as will be seen. The preparation used in Mahaux' studies was tested by Le Blond for the gonadotropic and the growth hormones and was found to contain only traces of these other anterior pituitary factors (personal communication).

EXPERIMENTAL CONDITIONS

In the investigation here reported healthy, young, male New Zealand white rabbits, weighing 2½ to 3½ kg., were used. As many factors as possible were controlled to insure correct measurements of the energy metabolism for the conditions imposed. For example, the feed[1] was constant in composition for all groups; but varied in quantity according to the influence produced by the several hormones upon the appetite of the animals. Food was regularly withdrawn from the experimental animal in the evening, at least 16 hours before he was to be placed in the apparatus next morning.

The necessary precautions as outlined by Lee (*loc. cit*), were carefully observed, except for the zone of thermal neutrality. It was found that the temperature recommended for the respiration chamber by Lee was too high for the small chamber available to us at the time this work was done. Therefore, the temperature of the constant temperature room, set originally at 30° C., had to be reduced to 25° C. to maintain the chamber at 30°. The difference in heat production, judged by the few experiments performed at 30° and the large number run at 25°, was negligible.

An open-circuit respiration apparatus was used. Outside air was pumped into a large metal spirometer, where it rapidly came to room temperature, then through the respiration chamber and past the sampling tubes. The latter were so suspended that the levelling bulb could be lowered by clock-work adjusted to the rate necessary to obtain a full sample in 1 hour. The samples were analyzed with a modified Haldane apparatus. Frequent samples of outside air were analyzed to test the reliability of the apparatus.

Certain alterations in the technical routine will be discussed in the several sections concerned with the separate hormones.

THE BASAL METABOLISM OF NORMAL RABBITS

A series of 21 determinations of the normal basal rate were conducted in strict accordance with modern concepts of the basal state. The average

[1] Purina Rabbit Chow.

of these determinations is accepted as the basal value of the heat production for normal rabbits in this study and all changes produced by the administered hormones are measured as variations from this average.

The normal basal figures appear in Table 1.

TABLE 1. AVERAGE METABOLISM OF THE NORMAL RABBIT IN THE BASAL STATE (21 determinations, expressed as Cal/M²/Hr.)[1]

Total calories	Non-protein cal.	Protein cal.	Non-protein R.Q.
34.8 $\sigma = 3.05$ $SE_M = 0.67$	27.3 $\sigma = 7.68$ $SE_M = 1.67$	7.5 $\sigma = 0.07$ $SE_M = 0.19$	0.839 $\sigma = .054$ $SE_M = .012$

Distribution of calories

Total calories			Percentage of total		
Carbohydrate	Protein	Fat	Carbohydrate	Protein	Fat
12.9	7.5	14.4	37	27	36

The figure for total heat production is within the range of values given by Smuts (1935), Benedict (1938) and Lee (1939).

[1] Smuts' formula for surface area of the rabbit, $Scm^2 = 56.33 \text{ Wt. gm.}^{.436}$ Arch. ges. Physiol. 232: 105, 1933.

THE METABOLISM OF RABBITS RECEIVING THYROTROPIC HORMONE

Ten male rabbits were injected subcutaneously with 75 guinea-pig units of thyrotropic hormone[2] per diem. Some of them were not used every week; but on others the metabolism was measured more than once on each of the 8 successive weeks of treatment, depending on the behavior of the animals in the maintenance of the basal state. The averages for the weekly periods are given in Table 2.

TABLE 2. EFFECTS OF THE THYROTROPIC HORMONE ON THE HEAT PRODUCTION OF THE NORMAL RABBIT IN TERMS OF CALS./M²/HR. (SMUTS' FORMULA)

Weeks	Total cal.	Non-prot. cals.	Non-prot. R.Q.	Distrib. of cals.			Per cent distrib. cals.			No. obs.
				Carb.	Prot.	Fat	Carb.	Prot.	Fat	
1	39.3	30.9	0.763	9.1	8.4	24.9	23	21	56	8
2	34.5[1]	27.1[2]	0.855[3]	13.7	7.4[4]	13.4	39	21	40	13
3	33.3[5]	27.2	0.846	13.8	6.1[6]	13.4	41	18	41	18
4	31.6[7]	26.7	0.824	10.7	4.9[8]	16.0	34	15	51	11
5	34.8[9]	29.0[10]	0.904	9.7	5.8	19.4	28	17	55	7
6	35.4	27.9	0.754[11]	4.5	7.4[12]	23.4	13	21	66	7
7 & 8	38.9[13]	30.5[14]	0.772	6.9	8.4[15]	23.6	18	22	60	7

P Values. That value numbered not different from preceding week.

[1] <0.01
[2] $<.05$
[3] $<.001$
[4] <0.4
[5] <0.2
[6] <0.1
[7] <0.1
[8] $<.02$
[9] $<.001$
[10] <0.2
[11] $<.05$
[12] Beyond limits of table
[13] $<.001$
[14] $<.05$
[15] <0.3

The average energy metabolism of the first week started 13% higher than the average normal; but in the fourth week it had fallen to 10% below, only to swing back in the fifth week when it was again at the normal level.

[2] Ayerst, McKenna Company, Montreal.

In the seventh week there were only 4 acceptable results and in the eighth week only 3. The average for the 7 experiments in the two weeks restores the level of the first week of treatment. Here is a variation of 23% from low point to high; which is not in general agreement with other reports concerning the metabolic effects of this hormone. According to Collip and Anderson (1934), Collip (1935), Thomson *et al.* (1941) an antithyrotropic hormone is produced, which, acting as an antibody, causes a refractory state to develop in the treated animal. In this regard the results here presented are in agreement up to the 4th week; but the subsequent rise is not accounted for in Collip's theory. Mahaux's reports on the rabbit (1938 a and b) show a tendency for the metabolism to return to the normal basal, after administration of thyroxin and thyrotropic hormone in sufficient quantity to suppress the effect of thyroxin on heat production. This feature of his work lends itself to the results recorded here which may be explained as follows (although Mahaux has a quite different theory).

Since the action of the thyrotropic hormone is upon the thyroid it follows that for the thyroid to maintain an increased activity as evidenced by these experiments, there must be new synthesis of hormone to match the accelerated rate of release. Let us suppose therefore that the gland responds to the hormone with a double action: (a) increased secretion producing the increased metabolism; and (b) increased synthesis of hormone, going on quietly during the recession period in the metabolism. It may further be reasonably supposed that the gland responds somewhat less sensitively to the stimulus for synthesis, or that this stimulus is slower in action that the stimulus for release of already stored thyroxin. Some time in the fourth week the effect of the hormone for synthesis approaches and exceeds the rate of release of thyroxin, thus providing more for release in the 5th and subsequent weeks, and a consequent higher rate of heat production. This theory seems to the authors to explain the facts brought out in these experiments quite as well as Collip's theory.

Looking at the energy sources supplying the calories along the course of the variations just noted in the total metabolism it is apparent that the low phase of energy production beginning with the second week is characterized by a readily available carbohydrate supply; but that from the fourth week on this declines to one-third of the level at two weeks and its place is taken by fat, up to the last two weeks, when it shows a reverse change. From the fourth week to the eighth inclusive fat metabolism dominates the picture. Protein metabolism falls off along with, but not exactly parallel with, the carbohydrate supply. Some of this decline might be attributed to the sparing effect of carbohydrate metabolism, but scarcely to this factor alone. As the food intake was not measured accurately in

these animals, it is impossible to assign other reasons for the decline unless we assume a depressant effect of the thyrotropic substance of the anterior pituitary, such as that reported by Mahaux (1938 a and b). However this may be, it is evident that the protein metabolism begins to rise again as the carbohydrate metabolism declines still farther.

THE METABOLISM OF INSULIN-TREATED ANIMALS

These rabbits were injected subcutaneously with one half unit insulin[3] per kg. of body weight. In order to calculate with a reasonable degree of accuracy the non-protein R.Q. of the animal in the respiration chamber without stopping the experiment for the purpose of collecting the urine by catheter, a comparison animal of the same sex and body weight, and fasted the same length of time, was kept in the bleeding box in the constant temperature room and the same dose of insulin was administered simultaneously with that given the animal to be placed immediately in the apparatus. Urine and blood samples were taken at hourly intervals post injection of insulin from this companion.[4] It was found that the nitrogen excretion from animal to animal was quite constant, if the comparison was made from the same hour of the day and calculated on the basis of the surface area. When such a scheme was carried out and the metabolism calculated as indicated the data in table 3 were secured.

TABLE 3. THE EFFECTS OF INSULIN ON THE RESPIRATORY METABOLISM ON NORMAL RABBITS

(Calorie values all in terms of Cal./M²/Hr.; each value in the Table is an average of sex determinations)

Hours after insulin	Total cal./M²/hr.	Non-prot. cal./M²/hr.	N.P. R.Q.	Distr. of cals.			Per cent distr.			Bl. sugar mgm./100
				Carb.	Prot.	Fat	Carb.	Prot.	Fat	
Basal	34.8[1]	27.8[2]	0.839[3]	12.9	7.5[4]	14.4	37	27	37	80
1st	35.5	27.7	.812	10.2	7.8	17.5	28.4	22	49.5	30
2nd	44.0	39.6	.844	18.7	4.4	20.9	42.5	10	47.5	30
3rd	41.0	33.0	.776	8.7	8.0	24.3	21.2	18.2	60.6	40
Col.	1	2	3	4	5	6	7	8	9	10

[1] SE_M =0.66 [2] 1.67 [3] 0.012 [4] 0.19

No alteration in the total or non-protein metabolism had taken place in the average result for six animals during the first hour after insulin, although the blood sugar dropped 50 mgs. per cent in this time. Nothing could more clearly illustrate the fact that a change in blood sugar does not in itself denote a change in the character of the foodstuff being oxidized (Hawley and Murlin, 1925). The respiratory quotient it will be observed fluctuated but little in the three hours. Sugar obviously had been with-

[3] Squibb's Regular Insulin.

[4] The data concerning urinary nitrogen excretion are included only for the effects of thyroxin (Table 4).

drawn from the blood and since but little apparently had been oxidized we would naturally surmise that it was laid down as glycogen. Twenty-four hours is sufficient to reduce the liver supply considerably and invite replenishment.

But there is other evidence that sugar is being utilized besides what we learn from the R.Q. In column 5 of the table it is evident that there had been a marked decrease in the average excretion of nitrogen; in other words, a marked sparing of protein amounting to 41% in the second hour after insulin. Coincident with this there was a sharp rise in the total metabolism and a still larger increase in the non-protein calories. The consequence is that in this same hour there was a 45% increase in both the carbohydrate and fat calories over the basal rate. Some of the sparing effect certainly is due to the increased combustion of carbohydrate as shown by a small increase in non-protein R.Q. But some of it also is probably accounted for by formation of glycogen as was most clearly shown by Cori (1924) in the effect of insulin on rabbits. Gaebler and Murlin (1925) also established with the phlorhizinized dog that when insulin was administered simultaneously with sugar the protein sparing effect was greater than when sugar alone was given.

If the urinary nitrogen excretion had not been determined every hour the protein sparing effect could not have been observed. It is noteworthy how the percentage of calories from fat (Col. 9) remains well above normal as the insulin effect wears off, while both the protein and the carbohydrate percentages are returning from their high and low points respectively.

THE SUPERMETABOLISM OF THE RABBIT PRODUCED BY THYROXIN

The animals used in this part of the study were injected subcutaneously once a week with one half milligram of crystalline thyroxin[5] per kg. of body weight. The respiratory metabolism was measured on the seven successive days after the day of injection (Table 4).

TABLE 4. SUPERMETABOLSIM OF NORMAL RABBITS PRODUCED BY THYROXIN
(All calorie values in terms of sq.M per hour)

Days after thyr.	Total cal.	N.P. R.Q.	Distr. of cals.			Per cent distr.			N exer. gms./M²/hr.	Bl. S. mgs. %	No. obs.
			Carb.	Prot.	Fat	Carb.	Prot.	Fat			
Basal	34.8	0.839	12.9	7.5	14.2	37	27	36	0.280	85	21
1	34.0	.838	12.8	6.9	14.3	38	20	42	.261	82	3
2	52.3	.784	11.6	8.0	32.7	22	15	63	.300	90	6
3	57.4[1]	.811[6]	17.4	10.3	29.7	30	18	52	.408	98	5
4	52.0[2]	.806	14.7	12.3	25.0	28	24	48	.464	106	8
5	52.0	.830	18.7	9.3	24.0	36	18	46	.313	109	3
6	47.2[3]	.818	15.7	8.3	23.2	33.4	17.6	49	.313	100	5
7	40.5[4,5]	.807	12.3	7.1	21.1	30.4	17.5	52.1	.268	94	8
8	37.0	.841	14.5	6.2	16.3	39.2	16.8	44	.234	81	3
Col.	1	2	3	4	5	6	7	8	9	10	11

P Values: That not significantly different from preceding day.
[1] <.01
[2] <.02
[3] <.02
[4] <.05
[5] From 4th Day <.001
[6] <.2

[5] Squibb's Crystalline Thyroxin.

The course of the energy production over these 8 days, inclusive of the day of administration of thyroxin, is in reasonable agreement with that obtained by other observers using the same animal subject, even though the data are not always expressed in the same terms (Mahaux, 1938 a and b; Le Breton, and Schaeffer 1935). Because the number of observations on the 2nd, 3rd, 4th, 6th and 7th days are more adequate for comparison with the basal values as established in table 1, we shall give most emphasis to these days. The low point in the non-protein R.Q. came in the second day, when the percentage of calories from fat reached its highest point. Since the protein metabolism had changed very little from the basal value at this stage of thyroxin effect, and the blood sugar had only just begun to rise, it is clear that we have here a true shift in the source of energy from carbohydrate to fat. On the third day the high point in the total heat production occurred, amounting to 65% above the basal. It is, incidentally, the high point in the response of the human subject also to an adequate dose of thyroxin. The cause may be ascribed in part to the specific dynamic action of protein metabolites; for it is clear that protein catabolism had now been accelerated (Col. 9). But the response of the muscles in particular to the thyroxin stimulus calls for support from carbohydrate and this evidently had become available; for both the blood sugar and the non-protein R.Q. had risen. It is probable that protein in part was the source of this sugar, i.e. it is a case of gluconeogenesis.

By the 4th day the protein metabolism had increased 77% over the first day and the blood sugar was still mounting, though not yet being oxidized at any faster rate. The high point of the total metabolism had now been passed, as had also the maximum rate of fat metabolism.

Going on to the 6th day protein metabolism had definitely declined, blood sugar was lower, fat metabolism still dominated the scene and total heat production had returned nearly to the level of the second day. All these regressive events were carried farther in the seventh and eighth days by which time the normal basal metabolism had virtually been reestablished.

The most abrupt change in all the events which we have observed, as the consequence of making available a great increase in thyroxin, occurred in the fat metabolism on the second day. The hormone appeared selectively to assault the fat stores of the body, for no other fat than was contained in the rabbit feed has been made available. Probably because the metabolism of fat does not protect the body protein as well as does carbohydrate metabolism the protein catabolism was increased by the fourth day, as we have seen to 77% over the first day. The abrupt change in fat metabolism on the second day amounted to 128%, while carbohydrate calories increased only gradually to the fifth day when the advance had become only 46% over the first day.

Another way of expressing the leading role of fat metabolism in the effects of thyroxin is to assemble the contributions of *extra calories* from the three foodstuffs day by day, starting from the first, which duplicated very nearly the average basal metabolism.

Days from 1st.	*2d*	*3d*	*4th*	*5th*	*6th*	*7th*	*8th*	*Sum*	*Ave.*
Extra Cal/M²/Hr. Cbh.	−1.2	4.6	1.9	5.9	2.9	−0.5	1.7	15.3	2.2
Extra Cal/M²/Hr. Prot.	1.1	3.4	5.4	2.4	1.4	0.2	−0.7	13.2	1.9
Extra Cal/M²/Hr. Fat	18.4	15.4	10.7	9.7	8.9	6.8	2.0	71.9	10.3

The average contribution from fat in calories per square meter per hour for the seven days was two and one-half times the contributions from both the other foodstuffs.

THE EFFECT OF THE COMBINED ADMINISTRATION OF INSULIN AND THYROXIN ON THE ENERGY METABOLISM OF NORMAL RABBITS

The animals were injected at weekly intervals with one-half mgm. of thyroxin per kg. of body weight per week and with one-half unit of regular

TABLE 5. THE SUPERMETABOLISM OF THE NORMAL RABBIT AS PRODUCED BY THYROXIN INJECTED ONCE A WEEK AND INSULIN ONCE ON EACH OF THE DAYS OF THE THYROXIN EFFECT[1]

(Calories are in terms of Cal./M²/Hr. and each value is a mean of the two effective hours of insulin)

Days after thyr.	Total cals.	N.P. cals.	N.P. R.Q.	Distr. of cals.			Per cent distr.			Ave. dif. in cal. bet. thy. & thy. +insulin
				Carb.	Prot.	Fat	Carb.	Prot.	Fat	
0[2]	46	34	0.805	12.0	12.0	22.0	26	26	48	11
1	58	49	.797	14.0	9.0	35.0	24.2	15.5	60.3	24
2	62	57.5	.785	15.5	4.5	42.0	25.0	7.2	67.8	9.7
3	58.6	55.4	.781	12.4	3.2	43.0	21.0	5.5	73.5	1.2
4	56	53	.769	12.0	3.0	41.0	21.5	5.3	73.2	4.0
5	51	48.5	.752	6.5	2.5	42.0	12.7	4.9	82.4	−1.0
6	49.5	44.4	.750	6.4	5.1	38.0	13.0	12.0	75.0	2.3
7	46	37.0	.767	6.5	9.0	30.5	14.1	19.6	66.3	5.5
8	42	33	.780	9.0	9.0	24.0	21.4	21.4	57.2	5.0
Col.	1	2	3	4	5	6	7	8	9	10

[1] There were two complete cycles after thyroxin, with insulin injection on each day, so that each value in the table is the average of four hourly determinations.

[2] This day is not a complete day of thyroxin effect, although insulin was given and a two-hourly respirarion experiment was run. The results consequently are essentially those of the insulin. Compare this day with the first in the experiments with thyroxin alone (Table 4). If insulin had been given on such a day, the results would have been the same as on the day labelled 0.

insulin per kg. just prior to making the metabolism measurement on each day of the week. As in the experiments with insulin alone a companion animal was kept in the bleeding box in the constant temperature room along side the respiration chamber in which the experimental animal was confined. Just before starting the first period both animals were bled and catheterized and were given equivalent doses of insulin. Both animals also were in the same stage of thyroxin treatment, i.e. in the same day after receiving the thyroxin injections. The nitrogen excretion in hourly periods by the animal in the bleeding box were used in calculating the energy

metabolism of the animal in the respiration chamber. This was checked against the total nitrogen excretion of the animal in the chamber for the total time of his confinement.

As will be seen the energy response of the rabbit receiving both hormones showed a two-phase reaction: first, the short-term effect of insulin in two hourly determinations; and second, the long-term weekly response to thyroxin. The summary data appear in table 5.

Analysis of the total heat production data indicates a peak over basal of 78% on the third day. Thyroxin alone produced on the corresponding day an increase of only 65% over the same basal. The difference of 13% is quite close to the average increase above basal (15.5%) for the three hourly determinations after insulin alone, as shown in table 3.

The mean weekly increase over basal metabolism (table 1) caused by the administration of the two hormones however, was 54%, while for thyroxin it was only 34%. The difference this time is 4.5% more than the increase over basal produced by insulin alone in the three hours after basal, as seen above.

Looking at the distribution of calories to the three foodstuffs, it is seen that there was a slight increase in absolute terms of the contribution from carbohydrate, continuing for only two days, and a very much greater increase in the contribution from fat which continued to the last days of the experiments. The summit of this increase for fat stands at 198% over basal and 95% over the starting level. The opposite trend in the protein calories continued to the fifth day when it amounted to 67% below basal (table 1) and 79% below the level of the first day recorded in table 5. Beyond this low point for protein, which happens to coincide closely with the low point for carbohydrate also, there is a gradual rise in both these contributions to the last day when they again became equal, as they were on the first day.

These changes, by and large, occur also in the percentage distribution of calories which roughly parallel those in the absolute distribution. The percentage of the total derived from fat remained high throughout, never returning to the original level. The relative contributions of protein and carbohydrate, on the contrary, began to decline on the first effective day and reached their low point once more at the high point for fat. Both carbohydrate and protein from their respective low points rose again and became equal on the last day, but at a lower level in the percentage scale.

We saw no such close parallelism between protein and carbohydrate calories under the influence of thyroxin alone. It would seem that the addition of the insulin influence must have synchronized the exposure of these two anti-ketogenic substances to the kindling enzymes at the seat of greatest combustion. It is worthy of note that insulin had its greatest

effect on the heat production in the first three days, counting the day of thyroxin injection (cf. tables 4 and 5, and col. 10 in table 5). On the first day when thyroxin became really and fully effective the insulin injection raised the metabolism 24 calories, or 70% over the first day of the thyroxin effect by itself. The next day it was only 9.7 calories, or 18%. Then for the succeeding four days there was hardly any effect, and finally on the last 2 days only 5 calories, or 13%. It is evident that there was some sort of supplementation between the two hormones in the first three days which did not recur again within the rest of the week. It should be remembered that the insulin effect referred to is the average effect of the two hours insulin was fully effective. In Fig. 1 may be seen, in a single cycle of the two hormones, how much more (except on the second day) insulin in the second hour affected the heat production, than in the first hour.

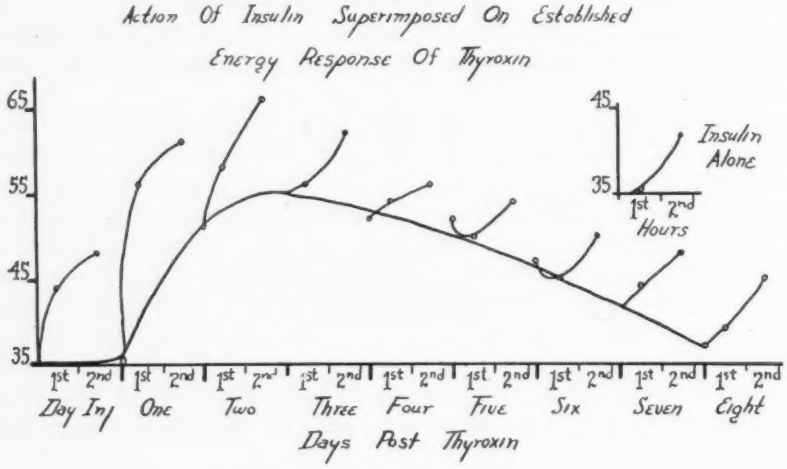

FIG. 1

This peculiar effect of insulin superimposed on the effect of thyroxin may be related to the frequent inhibitions by insulin of the gluconeogenic activity of the liver noted by Bach and Holmes (1937). Such an inhibition might well result in a decreased formation, coincident with increased utilization of, carbohydrate at the expense of the general body stores. Repeated inhibition of new formation combined with continued utilization under the stimulus of thyroxin would certainly deplete the most mobile forms of glycogen. A partial recovery from this condition apparent in the non protein R.Q's of the last days of the eight-day cycle (table 5) and still more plainly in the graph (Fig. 2) may be ascribed to an im-

proved tolerance to thyroxin and improved appetite produced by insulin, resulting in larger ingestion of the carbohydrate food.

DISCUSSION

The findings reported in this paper are, it is believed, based upon a sufficient number of experiments to give a reliable average for each of the

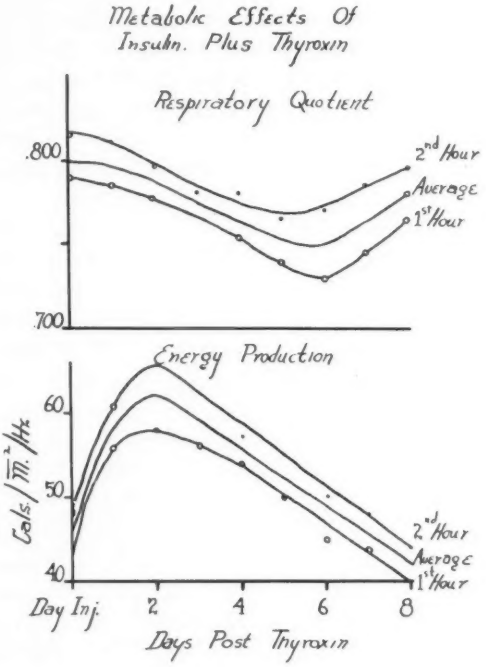

FIG. 2

conditions imposed upon the animals, and to give significant differences from preceding averages as shown by Fisher's "t" test. Exception has to be made of the results from the combination of insulin and thyroxin; for the experiments are too few in number to give reliable probability values. However, as noted above the average percentage excess heat production on the first three days caused by two hours of insulin added to thyroxin, closely approximates the average percentage increase over basal produced by insulin alone in the three hours shown in table 3; namely 15.5% (first hour scarcely raised the metabolism).

The experiments with insulin in this study were not designed to explain

how insulin produces its affect on heat production. It is well known, however, from the work of Cori and Buchwald (1930) on human subjects that the intravenous infusion of epinephrine at the rate of .00005 mg./Kg./min. produced not only a rise both in blood sugar and blood lactic acid but a rise in energy metabolism 13.6% (four subjects) above basal. This rate of dosage is only one-thirtieth of the maximal rate of discharge from the adrenal medulla, as measured by Cannon and Rapport (1921) following hypoglycemia produced by insulin administered to the cat. There is good reason therefore to infer that the average hypoglycemia of the rabbits as recorded in table 3 had by increasing the discharge of epinephrine produced at least a portion of the rise in heat production of the second hour, which amounted in the average to 26% above basal. The hyperglycemia which was just beginning in the third hour could not have furnished much fuel for combustion.

A few additional words should be said on the effect of thyrotropic hormone on energy production in the rabbit. The writers do not doubt the reality of antithyrotropic hormone discovered by Collip and Anderson (1934) and frequently confirmed by others. But the conditions of most of the experiments recorded by others except those of Mahaux on rabbits were quite different from those reported here. Collip and Anderson's observations on 3 rats extended only to the 46th day (for one only) and were treated with "large doses" of thyrotropic hormone. The 9 treated with serum of thyrotropic-resistant rats gave very variable results. Only two gave reductions below basal metabolism (-17 to -37%); others gave increases of $+14$ to $+17\%$ within the same 5 to 7 days and two maintained increases of 7 to 26% up to 10 to 15 days. None of the groups were very consistent one with another. So far as we have learned from the extensive literature reported by Thompson (1941) no one has carried out experiments in the same manner as ours. The simple explanation we have given on p. 78 seems to fit our results.

SUMMARY

In the normal basal state the New Zealand white male rabbit has a metabolic rate of 34.8 Cal/M²/Hr., with a SE_M of 0.67 and a characteristic distribution of calories supply.

The thyrotropic hormone influences energy production by virtue of its effect on the thyroid gland. The two-phase response over a period of eight weeks may be accounted for by a gradual depletion of the thyroid secretion, lasting four weeks, and a subsequent recovery to the normal rate of secretion, lasting another three weeks, after which the heat production becomes elevated again.

Insulin in a dose of $\frac{1}{2}$ U/Kg. produces an increased heat production

over basal in the first fully effective hour, of 26%, part of which may be attributed to induced epinephrine secretion.

Thyroxin in a dose of ½ mg. crystalline hormone increases energy production to a peak of 65% on the second day of its effect, with a mean value of 34% for the week. Throughout the week fat produces two and one half times as much extra metabolism as the other two foodstuffs combined.

Thyroxin once a week plus insulin every day increases the energy production to a peak of 78% on the second day with a mean of 54% for the week. There is a marked increase in the utilization of all energy sources. At the fifth day the cumulative effect of increased energy from both carbohydrate and fat has produced a protein-sparing effect of 79%. The marked supplementary effect of insulin which occurs the first three days does not recur again within the week.

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THE MECHANISM OF SYMPHYSEAL RELAXATION. THE DISTRIBUTION OF REDUCING GROUPS, HEXOSEAMINE, AND PROTEINS IN SYMPHYSES OF NORMAL AND RELAXED GUINEA PIGS[1]

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ALTHOUGH the presence of relaxin has been found to be a normal accompaniment of pregnancy in a number of species other than the guinea pig, only in the latter has a function definitely been assigned to this hormone. In an attempt to broaden our knowledge of possible areas of hormone-tissue interaction, a study has been made of the chemical changes occurring in the connective tissue of the guinea pig pubic symphysis during relaxation.

Comparative histological studies of the effects of estrogen, progesterone, relaxin, and combinations of these hormones have been made by Talmage (1947), who noted a reversible dissolution of cartilage and "breakdown" of connective tissue fibers in the pubic symphyses of guinea pigs treated first with estrogen and then with relaxin. He also observed a very considerable increase in alkaline phosphatase in symphyseal tissue following prolonged treatment with estrogen; the accumulation and distribution of phosphatase did not appear to be changed markedly upon relaxation.

Heringa and Van der Meer (1948) noted that the metachromasia characteristic of the symphysis of the non-pregnant female disappeared with the onset of relaxation during pregnancy, and reappeared following parturition and termination of the relaxed state. Similar changes were observed following experimental relaxation induced by injection of the serum of pregnant rabbits (PRS). These observations suggest at least two possible mechanisms as the chemical basis for relaxation. They may be summarized as follows: (1) Since the metachromatic reaction is considered to be more or less indicative of the presence of glycoproteins [e.g., hyaluronic acid; Wislocki *et al.* (1947)] it is possible that relaxation is accompanied (or preceded) by a depolymerization of glycoproteins of the ground substance of the pubic symphysis as suggested by Catchpole (1949). Such changes should result in alterations in the distribution of

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hexoseamine or reducing groups. Also, in this connection, studies of the hyaluronidase and glucuronidase contents of symphyseal tissue are of interest. (2) The dissolution of connective tissue fibers, as noted by Talmage, may indicate alteration either in the collagen or elastin of the tissue, or, more likely, alteration of the interfibrillar "cementing" matrix of connective tissue (Gross, 1949).

This paper reports the results of studies of the chemical changes which accompany relaxation of the connective tissue of the guinea pig pubic symphysis. Attention has chiefly been directed to changes in nitrogen, protein distribution, hexoseamine, and hydrolyzable reducing substance; some observations relative to hydrolytic enzymes are also included.

EXPERIMENTAL PROCEDURE

Approximately seventy animals were used in securing the data presented here. All were females, ovariectomized when the body weight reached 250–350 gm. Of these, twenty-five were large animals (800–900 gm.), which had been retired from the relaxin assay colony because of size, and had received no treatment of any kind for several weeks prior to their use in these experiments. The remainder were animals which had been set aside after castration, until their weights reached 400–500 gm., at which time each was used for a single experiment. In some instances, representatives of both groups were used in the same experiment, and the results were indistinguishable.

In order to obtain a sufficiently large tissue sample, all the animals were treated with either 1 or 2 micrograms of estradiol daily for periods ranging from two to eight weeks. For each set of experiments, about half the group was injected with relaxin; the rest served as estrogen-treated controls. Blood samples, when required, were taken by heart puncture immediately before autopsy. The animals were killed by a sharp blow on the head, and the symphyseal pads dissected out as discretely as possible, weighed, chilled to 4° C., and used within 3–4 hr.

Two samples of pregnant rabbit serum and two preparations of relaxin from ovaries of pregnant sows were used. The former were concentrated by the procedure of Albert and Money (1946), and preserved as dry powders; for both, 1.5–2 mg. represented one guinea pig unit. The two preparations from sow ovaries differed widely in potency; one (P-XLVI-1) was a relatively crude preparation, assaying about 15 units per mg., while the other (P-LI-17) assayed 100 units per mg.[2] Pfanstiehl trypsin (1:110) was used in one of the earlier experiments; later, crystalline trypsin (Armour) was employed.

[2] The methods used for assay of these preparations are described by Frieden and Hisaw (1950).

Reducing substance, after hydrolysis, was determined by the microprocedure of Folin and Malmros (1929). Hexoseamine was measured by the Elson and Morgan (1933) method. For total N, the samples were digested with selenium and sulfuric acid and the ammonia determined colorimetrically.

In all experiments, each symphyseal pad was minced, homogenized, and twice extracted with water or 0.9% NaCl; no difference could be noted between the two procedures. The solution and insoluble residue were studied separately. Appropriate experiments indicated that less than 3% of the total soluble N remained in the residue following two extractions.

I. *Effect of relaxin upon the water and N content of symphyseal connective tissue*

If a single injection of 5 units of relaxin is given to a guinea pig primed with estradiol as described above, there occurs, within 6 hours, a very

TABLE 1. EFFECT OF RELAXIN UPON WATER AND NITROGEN CONTENT OF SYMPHYSEAL TISSUE

Animal No.	E_{16}	E_{17}	R_{16}	R_{17}	R_{18}	R_{19}
Relaxin treatment	None		5 units PRS*		5 units relaxin**	
Pad weight, mg.	96.0	155.0	302	255	244	170
% water	78.1	76.5	82.8	83.5	81.1	80.0
% N, wet weight	2.93	2.93	2.08	2.06	2.09	2.35
% N, dry weight	13.4	12.5	12.1	12.5	11.1	11.8

These guinea pigs had been castrated for 4 months, and had received 2 micrograms estradiol daily for 45 days, prior to the experiment.
Relaxin-treated animals were killed 6 hours after injection.
* Pregnant rabbit serum.
** Preparation P-XLVI-1.

considerable increase in symphyseal pad weight averaging, in the experiments reported here, from 100–300%. The appearance of the symphysis changes from a white, tendon-like structure, to a more or less bloody and rather spongy mass. Generally speaking, the degree of relaxation as observed by palpation before autopsy, is correlated with the extent to which extravasation of blood has occurred. The fact that extravasation is rarely observed in control animals which had been similarly palpated indicates that this effect is not a result of manipulation.

Table 1 shows the total N and water contents of the symphyseal pads of six animals, four of which were relaxed; the other two had received only estrogen. It is apparent from consideration of the changes in weight and N content that only part of the increase in weight can be accounted for by influx of whole blood; the changes as observed are those to be expected if most of the fluid entering the symphysis were plasma. The increase in total weight and total N is quite variable. As shown in Table 2, the total

N of the unrelaxed symphysis ranges from 2.56–3.72%, averaging 3.08%, whereas the range of total N in the relaxed tissue is considerably greater. About 16% of the nitrogen of the unrelaxed symphysis may be removed by extraction with water or dilute salt solution. This figure appears to be subject to comparatively little fluctuation. As is to be expected, the increased N of the relaxed symphysis is completely soluble in these solvents.

TABLE 2. NITROGEN DISTRIBUTION SUMMARY

Condition of animal	Total nitrogen, % of wet weight			Soluble nitrogen, % of total		
	Average	Range	No. of detns.	Average	Range	No. of detns.
Unrelaxed	3.08 ± 0.24	2.56–3.72	23	16.5 ± 3.7	7.7–28.3	23
Relaxed	2.06 ± 0.32	1.41–3.37	31	40.1 ± 8.6	22.4–66.2	25

All guinea pigs had been treated with 2 micrograms estradiol daily for 3–7 weeks prior to the experiment.

II. *Distribution of reducing substance and aminohexose; Hyaluronidase and glucuronidase*

The unrelaxed guinea pig symphysis contains slightly more than 1 mg. of "reducing substance" (measured as "glucose" equivalents after hydrolysis) for each 100 mg. of wet tissue. Most of this is in a form other than "free" reducing sugar, i.e., it probably exists in combination with protein or as polysaccharide, and about half the total is insoluble in water or saline. Because of the uncertainty concerning the fluid entering during relaxation, both as to amount and composition, any comparison involving total carbohydrate content would be meaningless. Attention has been directed, therefore, toward study of the bound reducing substance and hexoseamine remaining in the residue after the water-soluble components have been removed. If the process of relaxation involved the depolymerization of a normally insoluble glycoprotein, it would be expected that the amount of water insoluble hexose[3] and hexoseamine would diminish following relaxation.

The water-insoluble residue was hydrolyzed over night at 100° in 4 N HCl, and reducing groups and hexoseamine, as well as total nitrogen, determined in the hydrolysate. The results have been expressed in terms of "reducing sugar": N or "hexoseamine": N ratios to facilitate comparison between tissues of different total weights.

The data are summarized in Table 3 in which are included results of

[3] While it is recognized that the Folin-Malmros procedure is not specific for hexoses it is assumed that the major contribution to reducing activity following the hydrolytic procedure used is probably a monosaccharide, and that changes in reducing activity most probably reflect changes in carbohydrate bound to protein.

determinations on 32 animals. Data for control and relaxed animals of a given group are bracketed together for convenience of comparison. It is apparent that, at least for the first three groups, there seem to be no consistent changes in ratios of either reducing sugar to residual nitrogen or hexoseamine to residual nitrogen upon relaxation.

TABLE 3. DISTRIBUTION OF HYDROLYZAEBLE HEXOSE* AND HEXOSEAMINE

Experiment No.	No. of animals	Relaxin treatment	Residue hexoseamine		Residue hexose*	
			Residue N		Residue N	
			Average	Range	Average	Range
1	4	None	.052 ± .011	.038–.072	0.25 ± .05	0.20–0.34
	3	4 Units PRS	.123 ± .058	.072–.210	0.57 ± .27	0.29–0.98
2	4	None	.088 ± .017	.069–.120	0.24 ± .04	0.20–0.32
	4	4 Units PRS	.076 ± .012	.055–.100	0.23 ± .01	0.22–0.24
3	6**	None	.041 ± .001	.039–.042	0.20 ± .01	0.19–0.21
	6***	4 Units PRS	.039 ± .005	.031–.044	0.16 ± .02	0.13–0.19
4	1 (E_{33})	None	.073		0.18	
	1 (R_{40})	10 Units P-LI-17	.051		0.17	
	1 (R_{41})	30 Units P-LI-17	.062		0.195	

The guinea pigs used for experiments 1 and 2, and some of those used in experiment 3, had been retired from the assay colony; the remainder had been castrated for 2–3 months. All animals had received 2 micrograms estradiol daily for 22–42 days prior to the experiment. PRS-treated animals were killed 6 hours after injection; P-LI-17 was administered 5 units q 6 hrs., and the animal killed 2–5 hours following the last injection.

* See footnote 3.

** Pads pooled in 2 groups.

*** Pads pooled in 3 groups.

Guinea pigs E_{33}, R_{40}, and R_{41} represent a series of three animals of similar size and history. E_{33} was a control. R_{40} received two injections of 5 units of relaxin at 4-hr. intervals, and was autopsied four hours after the second injection. It was found to be markedly relaxed. In R_{41}, although it had received six injections of 5 units of relaxin at 4-hr. intervals, and was killed about two hours after the last injection, relaxation was less pronounced. Within this last group of three animals, therefore, there appears to be a diminution of residue hexoseamine relative to insoluble nitrogen, as the extent of relaxation increases. This is the result to be expected from the hydrolysis or depolymerization of some hexoseamine-containing component of the "ground-substance" into smaller, more soluble units. In order to investigate this possibility more carefully, studies were made of the hyaluronidase and glucuronidase contents of "control" and relaxed symphyses. Extracts of both types of tissue were incubated with hyaluronate for 1–3 hr. at 37°; no detectable evidence of depolymerization could be obtained by the turbidimetric procedure. Since, under comparable conditions, nearly complete depolymerization can be demonstrated within 1 hour with 50 micrograms of hyaluronidase,[4] it may be concluded that hyaluronidase is present if at all in very low concentration.

[4] Schering, HD6C-1-7-III, 14 T.R.U./mg.

The β-glucuronidase content of symphyseal tissue appears to increase significantly during relaxation. These data will be reported in detail in another communication.

Studies of the amounts of acid and alkaline phosphatases of symphyseal tissue show that neither changes markedly upon relaxation, nor does relaxin exert any observable effect upon phosphatase *in vitro*.

III. *The effect of relaxation upon connective tissue proteins*

As previously noted, the appearance of the connective tissue fibers following relaxation suggests some possible alteration in either the collagen or elastin of the tissue. Pertinent to this possibility are the observations made by Gross (1949) who noted the presence in elastin fibers of a substance which could be digested by trypsin to leave behind a submicroscopic fibrillar residue. An effect by relaxin upon an analogous component of the connective tissue of the symphysis pubis might very well lead to disorganization of the type noted. Accordingly, studies of the effect of tryptic digestion upon the water-insoluble residues of control and relaxed symphyses were made. The results of preliminary studies indicated that the proportion of trypsin-resistant protein (collagen and elastin) was somewhat lower in the relaxed tissue. These studies were therefore extended and complemented by determination of collagen and elastin by the method of Lowry *et al.* (1941), slightly modified to accommodate the small quantities dealt with.

The water-insoluble residue was subjected to two successive extractions by 0.1 N NaOH for periods of 18 and 2 hr., respectively, at room temperature, and the nitrogen extracted by this procedure was determined. The alkali-insoluble residue, in two experiments, was suspended in water, the excess alkali neutralized, and the suspension autoclaved for a total of 8 hr. at 15 lbs. Since all but a negligible fraction of the total nitrogen of the alkali-insoluble residue is rendered soluble by this treatment, it may be concluded that all of this residue is collagen. In subsequent experiments, therefore, the last step was omitted.

In experiments with trypsin, the water-insoluble residue was suspended in an 0.2% solution of enzyme in phosphate buffer, pH 7.8–8.0 and incubated 18 hr. at 37°. The nitrogen released by this treatment is referred to as trypsin-soluble N. In two of the three sets of experiments reported here, the trypsin-insoluble residue was further extracted with alkali, whereupon an additional small (10–20%) amount of non-collagen N was extracted. The residue N is referred to as collagen N.

In what follows, in order to minimize nonspecific effects such as age, weight, length of estrogen treatment, etc., comparisons are generally drawn between groups of animals in which these factors are, as far as possible, identical.

It may be observed (Tables 4, 5, 6) that small but consistent differences are observed in the collagen content of the relaxed, as compared to the unrelaxed symphysis. The differences following a single injection of relaxin

TABLE 4. EFFECTS OF RELAXIN UPON THE COLLAGEN OF SYMPHYSEAL TISSUE

Animal No.	E_{18}	E_{19}	E_{20}	R_{20}	R_{21}	R_{22}
Body weight, gm.	660	735	625	615	780	650
Relaxin treatment	None			8 units relaxin**		
Pad weight, mg.	238	184	171	394	284	230
*Total N, mg.	7.24	5.60	5.20	13.85		4.70
Residue N, mg.	6.30	4.64	4.78	9.07		3.93
Trypsin-sol. N, mg.	1.73	1.31	1.29	3.01		1.45
Collagen N, mg.	4.57	3.33	3.49	6.06		2.48
% of residue N	72.6	71.8	73.0	66.8		63.1

These guinea pigs (except for R_{22}) had been retired from the assay colony. R_{22} had been castrated for 1½ months. All animals had received 2 micrograms estradiol daily for 31 days prior to the experiment. Relaxin-treated animals were killed 6 hours after injection.

* Calculated.

** Preparation P-XLVI-I.

TABLE 5. EFFECTS OF RELAXIN UPON THE COLLAGEN OF SYMPHYSEAL TISSUE

Animal No.	E_{21}	E_{22}	E_{23}	R_{23}	R_{24}	R_{25}
Body weight, gm.	610	625	470	750	570	600
Relaxin treatment	None			5 units PRS		
Pad weight, mg.	144	93	98	191	177	165
Total N, mg.	5.36	2.95	2.79	5.86	5.15	5.56
Soluble N, mg.	1.31	0.38	0.25	2.88	2.75	3.69
Residue N, mg.	4.05	2.57	2.54	2.98	2.39	1.87
NaOH-soluble N, mg.	1.22	0.82	0.91	0.93	0.79	0.70
Collagen N, mg.	2.83	1.75	1.63	2.05	1.60	1.17
% of residue N	69.9	68.1	64.2	68.8	67.0	62.6

These guinea pigs had been castrated for 2 months, and had received 2 micrograms estradiol daily for 51 days, prior to the experiment. Relaxin-treated animals were killed 6 hours after injection.

TABLE 6. EFFECTS OF RELAXIN UPON THE COLLAGEN OF SYMPHYSEAL TISSUE

Animal No.	E_{24}	E_{25}	E_{26}	R_{26}	R_{27}	R_{28}
Body weight, gm.	500	510	510	610	560	585
Relaxin treatment	None			5 units PRS		
Pad weight, mg.	60.0	75.0	95.0	215	189	182
Total N, mg.	1.94	2.24	2.72	3.99	3.53	3.92
Soluble N, mg.	0.34	0.27	0.50	1.12	1.10	1.20
Residue N, mg.	1.60	1.97	2.22	2.87	2.43	2.72
Trypsin-sol. N, mg.	0.51	0.60	0.77	1.01	1.16	1.08
NaOH-sol. N, mg.	0.15	0.11	0.10	0.13	0.10	0.13
Collagen N, mg.	0.94	1.26	1.35	1.73	1.17	1.51
Trypsin-sol. N, % of residue N	31.9	30.6	34.7	35.2	47.8	39.8
Collagen N, % of residue N	58.8	63.9	60.8	60.4	48.3	55.6

These guinea pigs had been castrated for 2½ months, and had received 2 micrograms estradiol daily for 43 days, prior to the experiment. Relaxin-treated animals were killed 6 hours after injection.

TABLE 7. EFFECTS OF RELAXIN UPON THE COLLAGEN OF SYMPHEAL TISSUE

Animal No.	E_{27}	E_{28}	R_{29}	R_{30}	R_{31}
Body weight, gm.	650	595	525	635	540
Relaxin treatment	None		30 units PRS		
Extent of relaxation	–	–	+++	++	+
Pad weight, mg.	104	110	430	351	249
Total N, mg.	3.15	3.02	6.65	6.07	4.46
Soluble N, mg.	0.48	0.55	3.20	1.86	1.00
Residue N, mg.	2.67	2.47	3.45	4.21	3.46
NaOH-sol. N, mg.	0.80	0.67	2.02	2.09	1.54
Collagen N, mg.	1.79	1.77	1.40	2.04	1.84
% of residue N	70.0	72.8	41.7	50.4	55.5

These guinea pigs had been castrated for 2½ months, and had received 2 micrograms estradiol daily for 45 days, prior to the experiment. Relaxin-treated animals were given 5 units q. 4 hr., and killed 6 hours after the sixth injection.

are more striking when studied by the tryptic digestion procedure, suggesting that in the early stages of the phenomenon there may be an alteration in sensitivity to trypsin without a concomitant increase in alkali-sensitivity. Subsequently, as indicated in Table 7, more striking alterations in alkali-sensitivity may supervene. The differences between relaxed and unrelaxed tissues observed following prolonged treatment with relaxin are considerably greater; furthermore, they may be correlated with the degree of relaxation as indicated by appearance at autopsy.

Table 8 summarizes data on a series of pigs, some of which had received relaxin or PRS at 4–5-hour intervals for 8–24 hours. The results of these experiments are essentially similar to those of the previous ones, except that the effects of relaxin as indicated by alkali extraction are less striking.

DISCUSSION

The results of the experiments described in this paper afford unequivocal support for neither of the hypotheses originally suggested as possible mechanisms for the action of relaxin. Changes in the glycoproteins of the ground substance, although apparently detectable histologically at

TABLE 8. EFFECTS OF RELAXIN UPON THE COLLAGEN OF SYMPHYSEAL TISSUE

Animal No.	E_{24}	E_{26}	E_{32}	R_{25}	R_{35}	R_{36}	R_{37}	R_{38}	R_{39}
Body weight, gm.	585	600	550	650	825	750	625	535	710
Relaxin treatment	None			10 units P-LI-17	20 units P-LI-17	30 units P-LI-17	10 units PRS	20 units PRS	30 units PRS
Extent of relaxa.	–	–	–	+	++	+	+	++	+
Pad weight, mg.	139	87.0	146	312	496	283	387	307	267
Total N, mg.	4.45	2.80	4.65	5.15	8.98	5.35	5.95	6.22	5.03
Soluble N, mg.	0.79*	0.50*	0.83*	1.50	3.06	1.59	2.13	2.91	1.79
Residue N, mg.	3.66	2.30	3.82	3.65	5.92	3.76	3.82	3.31	3.24
Trypsin-sol. N, mg.							1.56	1.29	1.17
NaOH-sol. N, mg.	0.98	0.49	0.82	1.18	1.89	1.04	0.13	0.12	0.11
Collagen N, mg.	2.68	1.81	3.00	2.48	4.03	2.72	2.13	1.90	1.97
% of residue N	73.2	78.7	78.5	67.7	68.1	72.4	56.0	57.4	60.8

These guinea pigs had been castrated for from 2 to 3 months, and had received 2 micrograms estradiol daily for from 40 to 60 days, prior to the experiment. Relaxin-treated animals were given 5 units q. 4 hr. and killed 2–5 hours after the last injection.

* Calculated.

an early stage in the process of relaxation, do not seem to be sufficiently extensive to be revealed by chemical techniques until much later. On the other hand, the data appear definitely to eliminate the possibility that relaxin affects an interfibrillar "cementing" matrix, since this would most reasonably be expected to result in a decrease, rather than an increase, in the proportion of the water-insoluble residue which is digestible by trypsin.

Our data are consistent with the assumption that the action of relaxin is exerted upon the collagen of the connective tissue of the symphysis, resulting in changes in the sensitivity of this protein to alkali and to trypsin. The effect upon trypsin-sensitivity appears to be most significant.

Gersh and Catchpole (1949) have suggested that relaxin may "directly or indirectly affect enzymes which cause breakdown of the connective tissue of the pubic symphysis," and report that extracts of the guinea pig symphysis, at certain stages of pregnancy, contain an enzyme which liberates dye from azocoll. The details of these experiments will be of considerable interest, although caution must be exercised in the interpretation of experiments dealing with such unnatural substrates.

If the action of relaxin is mediated by an enzyme, the most promising substrate available for its demonstration would seem to be the symphyseal tissue of a guinea pig sensitized by estrogen. Incubation of such tissues in extracts of relaxed symphyses failed to reveal any effects which could be demonstrated by the methods described above. Inasmuch as the enzyme content may depend upon the length of time the tissue is subjected to the action of relaxin, these studies are being broadened to vary the time factor, as well as to include the symphyseal pads of guinea pigs in various stages of pregnancy. The comparative effects of enzymes other than trypsin are likewise being examined.

Since this paper was written, there has appeared a report by Perl and Catchpole,[1] dealing with the changes occurring in the glycoproteins of the symphysis pubis upon relaxation. The conclusions reached by these authors are, in general, confirmed by our work; it would appear, however, that changes in the ground substance are detectable histologically at an earlier stage in the process of relaxation.

SUMMARY

Comparative chemical and enzymatic analyses of the connective tissue of control and relaxed guinea pig symphyses indicate that relaxin induces changes in the connective tissue proteins which are reflected in increased sensitivity of the latter to the actions of trypsin and of alkali. Significant

[1] Perl, E. and Catchpole, H. R.: *Archives of Path.* **50:** 233 (1950).

changes in carbohydrate- or hexoseamine-containing components (glycoproteins) are observed only following repeated administration of relaxin. Studies of the hyaluronidase and phosphatase contents of these tissues suggest that these enzymes play no significant role in the phenomenon of relaxation.

The relation of these facts to current theories regarding the role of relaxin is discussed.

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INFLUENCE OF HYPOPHYSECTOMY UPON THE DISTRIBUTION OF NORADRENALINE AND ADRENALINE IN THE SPLEEN OF THE RAT

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IT HAS been shown previously that administration of ACTH protein and peptides (Li 1948) in human patients brings about considerable alterations in the urinary output of adrenaline and noradrenaline (Euler and Luft 1949). The treatment caused a marked decrease in the absolute and relative amount of nor-adrenaline excreted by the kidneys whereas the adrenaline output was less influenced. The relative adrenaline figures consequently rose and sometimes reached figures of about 100 per cent of the excreted catechols as compared to some 30 per cent or less in normal urine.

Since these results constituted certain evidence that the catechol formation and excretion are influenced by disturbances in the endocrine balance and more especially in the ACTH-corticoid hormone system it appeared of interest to study the effect of hypophysectomy and ACTH-treatment on the distribution of the catechols in suitable organs. The spleen was chosen for this purpose since it is comparatively rich in catechols derived from its adrenergic nerve supply. A number of observations were also made on the catechol content of the suprarenals.

METHODS

Male white rats weighing 150–325 gm. were used. Hypophysectomy was made parapharyngeally in ether anaesthesia. The animals were maintained on a diet consisting of: casein 5%, wheat 68.5%, fish meal 10%, alfalfa leaf meal 10%, sodium chloride 1.5%, cod liver oil 5% and, in addition, fresh carrots and turnips twice a week and dried liver once a week. The animals were allowed to feed *ad libitum* and had free access to water.

The ACTH-preparations (kindly supplied by Dr. C. H. Li) were administered in the following way. The pure protein was given in doses of 0.5 mg. intraperitoneally in 0.5 cc. saline at 8, 16, and 23 hours during a period of 10 days. The peptides were given in three daily doses of 0.33 mg. during 9 days. The hypophysectomized control series RA VII and RA XII were given 0.5 cc. saline at the same hours.

The animals were killed with coal gas and the suprarenals, testicles and spleen carefully prepared and weighed. The suprarenals were extracted with some 10 cc. ethanol containing 1.25 cc. N-sulfuric acid per 100 cc. In order to prevent oxidation and red

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coloration, which otherwise appeared in the extracts prepared from the hypophysectomized animals, 1–2 mg. ascorbic acid was added to the alcohol. The spleens were extracted with 10% trichloracetic acid and the catechols adsorbed on alumina (Euler 1949). In series RA 65A and RA 65B 2 mg. ascorbic acid was added to the extraction fluid.

The catechol content of the suprarenal extracts was determined colorimetrically by the iodine method (Euler and Hamberg, 1949). Estimation of the catechols in the spleen extracts was carried out biologically (Euler 1949).

RESULTS

Rat spleen

Series	Number of animals	Total weight gm.	Total weight of spleen gm.	Adr. μg./gm. spleen	Noradr. μg./gm. spleen	Adr. μg./kg. animal	Noradr. μg./kg. animal	Per cent adrenaline	Weight of spleen per kg. animal
A. Controls									
R I	5	1160	4.95	0.09	0.41	0.38	1.75	18	4.3
RA VI	8	1400	9.6	0.07	0.24	0.49	1.65	23	6.9
RA XI	5	1555	5.04	0.07	0.48	0.23	1.55	13	3.2
RA 60	9	2200	8.4	0.097	0.57	0.37	2.08	15	3.8
RA 65B*	5	1260	7.0	0.07	0.33	0.39	1.83	18	5.6
B. Hypophysectomized (10 days R II, RA VII, RA XII, RA 37; 13 days RA 65A)									
R II	6	1260	5.9	0.14	0.36	0.64	1.66	28	4.7
RA VII	7	955	4.7	0.14	0.35	0.71	1.73	29	4.9
RA XII	5	1190	3.3	0.10	0.17	0.26	0.47	36	2.8
RA 37	7	1100	3.0	0.17	0.55	0.47	1.50	24	2.7
RA 65A*	6	1150	5.9	0.32	0.35	1.64	1.80	48	5.1
C. Hypophysectomized, ACTH treated	RA VIII 3×0.33 mg. peptides for 9 days RA 49 3×0.5 mg. protein for 10 days								
RA VIII	7	1005	4.2	0.06	0.45	0.26	1.88	12	4.2
RA 49	5	770	1.8	0.014	0.17	0.03	0.40	8	2.3

* 2 mg. ascorbic acid added to extraction fluid.

The effectiveness of the hypophysectomy was checked in every animal by controlling the weight of the testes and the suprarenals. All dubious cases were discarded.

From the table it follows that hypophysectomy in the rat is accompanied by a shift in the relative amounts of the catechols in the spleen. The increased adrenaline percentage in the hypophysectomized animals is due to an absolute increase in the adrenaline figure. Treatment of hypophysectomized animals with pure ACTH protein or peptides seems to bring the adrenaline percentage down to normal or even below. Although we have only two series of such animals, owing to lack of material, the figures indicate a specific effect of the ACTH preparations. The results therefore seem to warrant the conclusion that the disturbance in catechol balance caused by hypophysectomy may be restored by ACTH administration.

COMMENT

The results demonstrate that although the amounts of adrenaline found in the spleen are increased as a result of hypophysectomy the total catechol content is not significantly altered. The reason for the increase in adrenaline in the spleen in the hypophysectomized animals is not clear. Generally speaking it might be due to either an increased formation or a diminished utilization.

If one regards the latter possibility as more likely the effects of ACTH would be to promote the release of adrenaline, directly or indirectly, thereby lowering the content of adrenaline in the organ. A similar effect was noticed in the suprarenals of the ACTH treated animals. In 25 normal animals the suprarenal adrenaline was on an average 155 μg. per kg. body weight and after ACTH-treatment 113 μg. per kg. in 12 animals. Hypophysectomy did not appreciably alter the adrenaline content (145 μg. per kg. in 34 animals). The relative increase in adrenaline output in urine after ACTH-treatment in man, mentioned above, might be explained along the same lines.

The effect of ACTH on the distribution of catechols in organs supplied with adrenergic nerves and in the suprarenals raises the important question whether stress in various forms is accompanied by a shift in the formation and liberation of catechols in the adrenergic nerves and in organs supplied by such nerves.

SUMMARY

The relative and absolute adrenaline content of the rat's spleen was increased after hypophysectomy and restored to normal by treatment with ACTH (Li 1948) pure protein and peptides.

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EFFECT OF DIETARY FATS AND OILS ON ADRENAL CHOLESTEROL

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DURING a study of the anti-thyroid compound which occurs in rape seed (Carroll, 1949), it was observed that when rats were fed on diets containing high proportions of rape seed for two weeks or more, their adrenals were much whiter than those of normal rats. This effect had previously been noted by Kennedy and Purves (1941). Their histological studies indicated hypertrophy of the adrenal cortex with a great increase in the lipoid content of the cortical cells.

In the experiments to be described it will be shown that the changes produced in the adrenal cortex are independent of the anti-thyroid activity of rape seed and are produced mainly by a factor contained in the seed oil. The results obtained by feeding a variety of other oils and fats are also presented.

MATERIALS AND METHODS

Young male rats of the Sprague-Dawley strain were used in these experiments unless otherwise indicated. The individual fats, oils or rape seed fractions were intimately mixed with the regular diet of Masters' fox chow. This chow contains a minimum of 20% protain, 3.5% fat, and a maximum of 5% fibre. In the experiments in which hypophysectomized rats were used, the oil was mixed with a synthetic diet consisting of 37.3% corn starch, 24.2% glucose, 5.7% lard, 30.0% casein and 2.8% of Steenbock's salt mixture No. 40, together with an adequate vitamin supplement.

The rape seed (Brassica Napus) was Dwarf Essex variety.[2] Fractions from this seed were prepared as described previously (Carroll, 1949). Briefly, the seed was first extracted with ether which removed the seed oil (25–30%). The residue was then extracted with water and finally this aqueous solution was extracted continuously with ether, which removed only a small fraction (less than 1% of the total seed), but which contained practically all of the anti-thyroid compound. This compound was further purified and was shown to possess the same properties as L-5-vinyl-2-thiooxazolidone isolated by Astwood, Greer and Ettlinger (1949). For all experiments except that described in Table 1, a commercial rape oil was used.[3] This oil was pressed from seed grown in Western Canada,

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[1] Holder of Medical Research Fellowship administered by the Canadian Life Insurance Officers Association.

[2] This seed was obtained from W. H. Perron and Co. Ltd., Montreal.

[3] The rape oil was very generously supplied by Yocum Faust Ltd., London, Ontario. The author is also indebted to Dawe's Vitamins Inc., Chicago, for the sample of

TABLE 1. EFFECT OF RAPE SEED FRACTIONS ON RAT ADRENALS

Fraction	Per cent in diet	Rat weight		Thymus weight (mgm.)	Adrenal weight (mgm.)	Adrenal ascorbic acid		Adrenal cholesterol		
		Initial (gm.)	Final (gm.)			mgm./100 gm. of adrenal weight	absolute[1] amount (mgm.)	Free (mgm./100 mgm. of adrenal weight)	Total (mgm./100 mgm. of adrenal weight)	absolute[1] amount (mgm.)
Control	—	90±1.9	193± 5.5	344±24	30.9±0.47	445±18	0.138	0.22±0.05	4.1±0.64	1.27
Whole Rape Seed	45	85±1.3	73± 1.8	69± 5.4	34.2±0.99	357± 9.4	0.122	0.76±0.04	11.3±0.90	3.84
Ether soluble (rape oil)	25	84±2.6	151±11	323±47	38.5±2.6	378±22	0.146	0.40±0.06	9.2±1.1	3.54
Ether insoluble	35	68±1.3	73± 5.1	104±25	24.3±0.8	333±17	0.081	0.50±0.03	8.7±1.5	2.12
Continuous ether extract (containing anti-thyroid compound)	2	67±1.3	56± 1.4	43± 9.9	18.6±4.3	423±18	0.079	—	8.2±0.7	1.52
Anti-Thyroid compound (L-5-vinyl-2-thiooxazolidone)	0.2	81±2.1[2]	125± 2.5	241± 5.6	22.4±1.06	—	—	—	7.6±0.26	1.70

Diets were fed for 4 weeks. Results are given as Average Value ± Standard Error.
[1] Absolute amount = percentage of ascorbic acid or cholesterol × weight of the paired adrenals.
[2] Four rats in this group. Other groups contain 6 rats.

TABLE 2. EFFECT OF VARIOUS FATS AND OILS ON RAT ADRENALS

Fat or oil	Iodine number	Saponification number	Rat weight		Thymus weight (mgm.)	Adrenal weight (mgm.)	Adrenal cholesterol		
			Initial (gm.)	Final (gm.)			Free (mgm./100 mgm. of adrenal weight)	Total (mgm./100 mgm. of adrenal weight)	absolute[1] amount (mgm.)
Control	—	—	80±2.5	178±4.2	507±41	28.9±0.56	0.22±0.05	4.6±0.61	1.33
Turnip Seed Oil	109	176	86±1.0	151±7.9	198±27	41.4±1.3	0.68±0.11	13.5±0.63	5.59
Rape Oil	103	175	78±2.9	169±4.0	341±27	36.2±1.2	0.69±0.04	14.1±0.69	5.10
Cod Liver Oil	161	186	84±2.6	137±9.9	285±29	27.4±0.7	0.37±0.05	10.7±0.38	2.94
Olive Oil	87	191	86±4.9	194±6.7	386±19	34.1±1.7	0.36±0.01	8.1±0.50	2.76
Butter Fat	33	193[2]	84±2.7	194±4.0	403±12	31.4±1.2	0.49±0.04	7.4±0.55	2.32
Cottonseed Oil	107	195	88±2.6	155±5.1	260±19	27.9±1.7	0.30±0.05	8.0±0.45	2.23
Corn Oil	127	190	85±1.8	164±1.4	284±32	25.8±0.6	0.32±0.06	7.0±0.27	1.81
Linseed Oil	181	192	72±2.9	144±5.9	328±26	24.8±1.1	0.54±0.02	6.8±0.57	1.69
Sesame Oil	109	194	84±3.5	135±7.9	260±33	24.8±1.6	0.33±0.05	6.7±0.42	1.66
Margarine	63	168[2]	63±2.7	147±14	353±28	25.1±2.8	0.42±0.03	5.6±0.20	1.40
Dogfish Oil[3]	106	158	75±1.2	103±1.5	192±7.7	23.5±0.3	0.39±0.11	4.4±0.68	1.04

Diets were fed for 4 weeks. All fats or oils were fed as 25% by weight of the diet.
Results are given as Average Value ± Standard Error.
[1] Absolute amount = percentage of cholesterol × weight of the paired adrenals.
These values are lower than expected, perhaps because of added materials in these commercial products.
[2] Four rats in this group. Other groups contain 6 rats.

and had been alkali-refined and bleached. The turnip seed oil consisted of the ether-extractable material from Swede turnip seed (Brassica napobrassica—variety Canadian Gem).

The rat adrenals were removed under nembutal anesthesia for analysis. One adrenal of the pair was used for the cholesterol determinations. It was extracted three times with 3 cc. portions of boiling acetone-alcohol (1:1), and the total volume made up to 10 cc. and filtered. Free and total cholesterol was estimated on 5 and 2 cc. aliquots respectively by a modification of the method of Sperry and Schonheimer (Hawk, Oser and Summerson, 1947). Adrenal ascorbic acid was measured by the method of Roe and Kuether (1943) and adrenal phosphorus by the method of Allen (1940).

Iodine numbers of the oils were determined by the Hanus method (Woodman, 1941). Saponification numbers were determined as described by Woodman (1941).

RESULTS

In the initial experiment, the various fractions prepared from rape seed were fed to rats for periods of four weeks and their effect on adrenal size and adrenal cholesterol was measured. These results are presented in Table 1.

It can be seen that feeding rape seed as 45% by weight of the diet produced a slight increase in adrenal size and a very marked increase in both the percentage and the absolute amount of adrenal cholesterol. The seed oil produced nearly the same effect when fed as 25% of the diet, and the adrenals had the same white appearance which was noted when the whole seed was fed. The other fractions tended to increase the percentage of total adrenal cholesterol, but because the adrenals were much smaller, the absolute amount was not increased to nearly the same extent.

It is interesting to note that rats failed to gain weight on a diet containing 45% rape seed and that this effect was also produced by feeding the continuous ether extract containing the anti-thyroid compound as only 2% of the diet. On the other hand, rats grew much better on a diet containing 25% rape oil, although they still grew less well than controls. Rats on rape oil diets usually became quite dirty and greasy, and occasionally showed a tendency to lose fur from the back of the neck.

In order to see whether the effect on adrenal cholesterol produced by rape oil was specific to that particular oil, a variety of other fats and oils were fed as 25% by weight of the diet to groups of 6 rats for periods of four weeks. The results of these experiments are shown in Table 2. The oils are arranged in order of their ability to increase adrenal cholesterol.

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dog fish oil (Squalus sucklii)—5130 I.U. Vitamin A per gram. The other oils were obtained as follows: cod liver oil—1800 I.U. Vitamin A and 180 I.U. Vitamin D per gram (E. R. Squibb & Co.), olive oil—Ballardvale refined (Rexall Drug Co.), sesame oil and cottonseed oil—Ingram and Bell, Montreal, corn oil (Mazola)—Canada Starch Co. Ltd., linseed oil—Sherwin-Williams Paint Co., Ltd.

[illegible] values are lower than expected, perhaps because of added [illegible]
[illegible] Four rats in this group. Other groups contain 6 rats.

Although the adrenal cholesterol was increased above that of the controls with nearly every fat or oil tested, by far the greatest effect was produced by feeding rape oil or turnip seed oil. These two oils might be expected to act similarly since they are derived from plants belonging to the same family (Cruciferae) and have a similar composition. It is notable that while rape oil and turnip seed oil produced a definite increase in adrenal size,

TABLE 3. RATE OF ACCUMULATION AND PERMANENCE OF STORED ADRENAL CHOLESTEROL

Time on diet containing 25% rape oil (days)	Time on fox chow diet (days)	No. of rats	Rat weight		Thymus weight (mgm.)	Adrenal weight (mgm.)	Adrenal cholesterol		
			Initial (gm.)	Final (gm.)			Free	Total	
							(mgm./100 mgm. of adrenal weight)	(mgm./100 mgm. of adrenal weight)	absolute[1] amount (mgm.)
					Treated				
7	—	6	85±0.78	107±4.9	267±28	22.9±0.76	0.45±0.07	7.0±0.56	1.60
14	—	6	85±0.45	126±2.6	354±29	30.1±0.82	0.49±0.06	11.7±0.67	3.53
28	—	24	82±1.2	157±3.6	303±21	37.5±0.81	0.55±0.04	12.0±0.65	4.50
61	—	2	90	250	—	47.3	0.47	14.0	6.62
98	—	3	91	289	—	59.4	0.52	11.0	6.54
190	—	3	90	373	226	56.5	1.55	12.2	6.90
26	—	6[2]	89±2.8	139±4.9	340±31	48.6±2.9	0.57±0.07	13.5±1.6	6.56
					Controls				
—	7	6	85±0.68	111±2.7	329±21	21.2±0.76	0.40±0.09	3.0±0.30	0.64
—	14	6	85±0.68	153±5.1	510±45	29.8±1.8	0.33±0.03	3.4±0.28	1.02
—	28	24	84±1.3	189±3.1	417±23	30.2±0.41	0.32±0.05	4.4±0.28	1.33
—	61	2	86	284	—	32.5	0.25	5.5	1.79
—	98	3	85	319	—	37.5	0.35	5.1	1.91
—	190	3	83	390	249	41.0	0.42	4.4	1.81
—	26	6[2]	89±2.8	149±5.9	357±35	34.7±1.9	0.46±0.04	6.0±0.31	2.08
					Treated				
30	3	2	88	197	433	43.2	0.63	11.3	4.89
30	7	2	87	224	525	44.7	0.55	11.4	5.10
30	14	2	86	223	292	48.0	0.48	12.2	5.86
26	21	3	80	229	307	41.7	0.40	9.9	4.13
28	28	5	91	262	358	40.4	0.32	6.9	2.79
					Controls				
—	33	2	95	215	502	28.7	0.38	4.9	1.41
—	37	2	95	233	551	34.4	0.58	4.2	1.45
—	44	2	88	252	435	32.4	0.40	4.6	1.49
—	47	3	97	231	215	30.5	0.10	4.3	1.31
—	56	5	89	272	326	37.9	0.24	4.3	1.63

Results are given as Average Value ± Standard Error.
[1] Absolute amount = percentage of cholesterol × weight of the paired adrenals.
[2] Female rats were used.

most of the other oils and fats tended to decrease the size of the adrenals.

The rate of accumulation of cholesterol in the adrenals has been studied by sacrificing groups of rats after varying periods of time on a diet containing 25% rape oil. Other rats have been fed this rape oil diet for periods of approximately four weeks, and then changed to a fox chow diet. Groups of these rats were sacrificed from time to time to determine the rate of loss of the increased store of adrenal cholesterol. These results are presented in Table 3.

It appears that the adrenal cholesterol reached a maximum concentration after two weeks on the rape oil diet and did not increase further over a six months period. However, the increase in adrenal size over that of controls began to appear only after one month on the diet. In the rats

which were changed from a rape oil diet back to a straight fox chow diet, the concentration and total amount of adrenal cholesterol began to fall only after three weeks.

The results given in Table 3 for groups of female rats indicate that a rape oil diet produced an increase in adrenal cholesterol similar to that observed in male rats.

Although the amount of adrenal cholesterol was greatly increased in rats fed on rape oil, such changes were not observed in the adrenal ascorbic acid (Table 1) and the adrenal phosphorus (Table 4). The average concentration of both of these components was lower in rats fed on rape oil, but since their adrenals were larger, the absolute amounts were nearly the same as in control rats.

TABLE 4. EFFECT OF RAPE SEED DIET ON ADRENAL PHOSPHORUS

	Rats on fox chow diet	Rats on diet containing 25% rape oil
Days on diet	29	29
Number of rats	6	6
Initial weight (gm.)	83 ± 2.3	83 ± 1.2
Final weight (gm.)	195 ± 2.6	145 ± 4.2
Adrenal weight (mgm.)	29.3 ± 0.53	36.6 ± 1.2
Total adrenal cholesterol (mgm./100 mgm. of adrenal weight)	5.2 ± 0.61	9.7 ± 0.73
Adrenal phosphorus (mgm./100 gm. of adrenal weight)	154 ± 7.3	141 ± 6.6
Absolute amount[1] (mgm.)	0.045	0.052

Results are given as Average Value ± Standard Error.
[1] Absolute amount = percentage of phosphorus × weight of paired adrenals.

The effect of a rape oil diet on the cholesterol content of the blood and of various tissues other than the adrenal has been studied, and the results are presented in Table 5. It may be seen that of the organs tested, only the liver showed a marked increase in cholesterol content, and there, as in the adrenal, nearly all of the increase was due to ester cholesterol.

Feeding rape oil to hypophysectomized rats did not cause a significant increase in their adrenal cholesterol, and the adrenals atrophied as is usual in such rats. On the other hand, when rats were first fed rape oil for four weeks and then hypophysectomized and continued on the rape oil diet; although their adrenals atrophied, the amount of cholesterol which they contained remained high, and the concentration of adrenal cholesterol reached the highest values observed during this study. Table 6 contains the results of these experiments.

The method used for measuring cholesterol in tissues is not entirely specific for this substance, and it seemed desirable to investigate more thoroughly the nature of the increased lipid in the adrenals of rats fed on

TABLE 5. EFFECT OF RAPE OIL DIET ON THE CHOLESTEROL CONTENT OF VARIOUS ORGANS

	Rats on fox chow diet	Rats on diet containing 25% rape oil
	Experiment 1	
Days on diet	26	26
Number of rats	6	6
Initial weight (gm.)	84 ±2.3	84 ±2.4
Final weight (gm.)	191 ±9.7	162 ±3.9
Adrenal weight (mgm.)	31.7 ±1.2	38.7 ±1.2
Cholesterol Free (%)	0.42 ±0.07	0.55 ±0.05
Total (%)	3.7 ±0.3	14.4 ±0.81
Liver weight (gm.)	7.88 ±0.46	8.08 ±0.26
Cholesterol Free (%)	0.19 ±0.02	0.19 ±0.01
Total (%)	0.22 ±0.01	0.52 ±0.05
Kidney weight (gm.)	1.52 ±0.11	1.32 ±0.05
Cholesterol Free (%)	0.34 ±0.04	0.41 ±0.02
Total (%)	0.43 ±0.02	0.48 ±0.03
Lung weight (gm.)	1.10 ±0.07	1.21 ±0.18
Cholesterol Free (%)	0.38 ±0.04	0.35 ±0.05
Total (%)	0.49 ±0.04	0.49 ±0.05
Brain weight (gm.)	1.63 ±0.03	1.42 ±0.13
Cholesterol Free (%)	1.54 ±0.37	1.48 ±0.08
Total (%)	1.61 ±0.06	1.65 ±0.01
Retroperitoneal Fat Cholesterol Total (%)	0.061 ±0.008	0.085 ±0.003
	Experiment 2	
Days on diet	28	28
Number of rats	5	6
Initial Weight (gm.)	86 ±0.74	85 ±1.0
Final Weight (gm.)	194 ±4.1	170 ±5.9
Blood Cholesterol		
Free (mgm./100 cc. plasma)	20 ±2.0	27 ±3.1
Total (mgm./100 cc. plasma)	84 ±7.3	96 ±7.7
	Experiment 3	
Days on diet	61	61
Number of rats	2	2
Initial Weight (gm.)	86	90
Final Weight (gm.)	284	250
Heart Weight (gm.)	0.870	0.773
Cholesterol Total (%)	0.120	0.116
Spleen Weight (gm.)	0.765	0.561
Cholesterol Total (%)	0.31	0.37
Testis Weight (gm.)	3.27	3.11
Cholesterol Total (%)	0.15	0.16

Results are given as Average Value ±Standard Error.
All cholesterol values are expressed as per cent of wet weight of the tissue.

rape oil. Accordingly, two groups of 6 rats each were kept on diets of fox chow and fox chow plus 25% rape oil respectively, for one month. Then the animals were sacrificed and the adrenals of each group were pooled. They were extracted 5 times with 5 cc. portions of boiling acetone-alcohol (1:1). The solvent was removed in vacuo and the residue taken up in 5 cc. of acetone-alcohol (1:1), 15 drops of 30% aqueous potassium hydroxide was added, and the solution allowed to stand at 37° C. for 30 minutes.

TABLE 6. EFFECT OF RAPE SEED OIL DIETS ON HYPOPHYSECTOMIZED RATS

Days on diet			Rat weight		Thymus weight (mgm.)	Adrenal weight (mgm.)	Adrenal cholesterol		
							Free	Total	
Before hypophysectomy	After hypophysectomy	No. of rats	Initial (gm.)	Final (gm.)			(mgm./100 mgm. of adrenal weight)	(mgm./100 mgm. of adrenal weight)	absolute[1] amount (mgm.)
29	26[2]	5	91±6.4	124±7.8	101±3.7	20.4±0.98	1.04±0.02	17.6±0.92	3.59
0	24[3]	4	159±8.3	126±5.8	72±6.9	11.1±0.81	—	3.8±0.29	0.42
0	24 (controls[4])	4	151±8.0	149±4.8	243±18	12.9±0.62	—	3.3±0.93	0.43

Results are given as Average Value ± Standard Error.
[1] Absolute amount = percentage of cholesterol × weight of the paired adrenals.
[2] The rape oil was added to fox chow before hypophysectomy and to the synthetic diet after hypophysectomy.
[3] This group were started on the rape oil diet on the second day following hypophysectomy.
[4] This group were fed the synthetic diet without added rape oil.

Then the non-saponifiable and fat-acid fractions were recovered by extraction with ether. The results of these extractions are shown in Table 7. It is evident that the increased weight in the adrenals of the treated rats is more than accounted for by that fraction soluble in acetone-alcohol.

TABLE 7. ADRENAL EXTRACTIONS

	Rats on fox chow diet	Rats on diet containing 25% rape oil
Pooled adrenal weight (mgm.)	169.7	205.6
Acetone-alcohol soluble (mgm.)	21.7	63.0
Acetone-alcohol insoluble (mgm.)	148.0	142.6
Non-saponifiable (mgm.)	8.8	30.7
Fat acid (mgm.)	10.6	21.3

The ultraviolet absorption spectrum of the nonsaponifiable fraction from the treated adrenals corresponded closely with that of cholesterol. This fraction was recrystallized from alcohol and the purified material was identified as cholesterol by melting point and mixed melting point with an authentic sample. Since the cholesterol normally present in adrenals could account for only one-third or less of this fraction, these results are interpreted as meaning that the lipid material which accumulates in the adrenals as the result of rape oil feeding is actually cholesterol. Most of this additional cholesterol is esterified, but increases are also observed in free cholesterol. In agreement with the findings of Kennedy and Purves (1941), our histological studies have shown that this lipid is confined to the adrenal cortex.

DISCUSSION

Several laboratories have described changes in the adrenal gland produced by alterations in the diet. It has already been noted that Kennedy and Purves (1941) found adrenal hypertrophy following the feeding of rape seed diets. Ingle (1945) reported adrenal hypertrophy in rats fed on

high casein and lactalbumin diets in which approximately 80 per cent of the caloric value of the diet was derived from protein. Of more interest in the present discussion is the work of Abelin (1948) on the effect of fats in the diet. He found that feeding rats a diet containing 12 per cent "Speise oil" (a mixture containing rape oil, peanut oil and poppyseed oil) for ten to twelve days caused an increase in the concentration of adrenal cholesterol to 6%. Other fats such as pork fat, cocofat, bone marrow and oils such as almond oil and fish oil failed to produce this effect.

The general subject of adrenal hypertrophy has been reviewed by Tepperman, Engel and Long (1943). It is evident that the size and cholesterol content of the adrenal cortex may be altered in a great variety of circumstances. However, many of the causative agents may be classed as stresses to the body, all of which, regardless of their nature, elicit a characteristic response. Part of this response appears to be increased liberation of adrenocorticotrophic hormone and consequent changes in the adrenal cortex. A sudden temporary period of stress (e.g. hemorrhage) produces an initial lowering of adrenal cholesterol followed by a rise to above normal during the recovery period. A gradual change in the environment (e.g. fasting) produces a gradual increase of adrenal cholesterol. The adrenals hypertrophy in both of these instances. These changes have been discussed by Sayers and Sayers (1948).

The increases in adrenal cholesterol produced by rape oil feeding are much greater than those attained in response to stress. Further, rats fed on this diet continue to gain weight, although less rapidly than controls, and their average thymus weight approaches that of controls. These facts lead one to believe that some other mechanism than a response to stress is responsible for the observed changes in their adrenals. Rape oil fails to increase the adrenal cholesterol in hypophysectomized rats, but in such animals the metabolism of the adrenal is reduced to a very low level and even factors not directly controlled by the adrenocorticotrophic hormone may fail to produce their effects in its absence.

The increases in adrenal cholesterol observed in the rats fed other fractions of rape seed may, on the other hand, be at least partially the result of a response to the stress occasioned by the unusual diet. In some instances the animals apparently starved themselves because of distaste for the diet, as reflected in their failure to gain weight. This, in itself, would constitute a stress.

Cholesterol has been suggested as a precursor of the adrenocortical hormones (Long 1947) in view of their common steroid nature and because of its high concentration in the adrenal cortex. While there is no direct evidence that this hypothesis is correct, the concentration of adrenal cholesterol drops markedly after administration of adrenocorticotrophic

hormone (Sayers, Sayers, Fry, White and Long 1944) and after various forms of stress (Sayers and Sayers 1948), and at the same time there is evidence of increased secretion of adrenal cortical hormones. At later stages during the response to stress, the concentration of adrenal cholesterol may increase to above normal while the animal still shows evidence of increased adrenal hormone secretion.

Experiments have thus far failed to indicate marked hyper- or hyposecretion of hormones from the adrenals of rats fed on rape oil diets. Further experiments are being performed on this phase of the problem as well as studies on the nature of the substance in rape oil responsible for the observed effects.

SUMMARY

The increase in size and lipid content of rat adrenals produced by feeding a diet containing rape seed has been found to be the result of cholesterol deposition caused by a factor in the seed oil. The adrenal cholesterol is increased to three to four times the normal amount when this oil is fed as 25 per cent by weight of the diet for four weeks. The increased cholesterol is mostly in the esterified form, and is confined to the adrenal cortex. The same effect may be produced by feeding Swede turnip seed oil. Various other fats and oils fed at the same level in the diet tend to increase the amount of adrenal cholesterol to a much smaller degree.

While the concentration of ascorbic acid and of phosphorus in the adrenal was somewhat lower in rats fed rape oil, the absolute amount was nearly the same as in control rats.

A diet containing rape oil also caused a marked increase in the esterified cholesterol content of the liver, but not in various other tissues nor in the blood.

The effect on adrenal cholesterol produced by this diet was not observed in hypophysectomized rats.

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NOTES AND COMMENTS

FAILURE OF GROWTH HORMONE TO PROMOTE A WEIGHT INCREMENT IN IMMATURE RATS UNDER CONDITIONS OF LOW ENVIRONMENTAL TEMPERATURE[1]

Available data indicate that prolonged exposure to cold results in a marked increase in thyrotrophin (Brolin, 1946) and cort'cotrophin (Sayers and Sayers, 1945) production. Somatic growth, however, is inhibited. It has been suggested that the retarded growth under conditions of low environmental temperature is due, at least in part, to a "shift in pituitary-hormone formation" resulting in an increased production of the life-maintaining corticotrophin (and possibly other corticotrophic hormones) at the expense of the less urgently needed somatotrophin (Selye, 1946). If this were so, then providing increased amounts of somatotrophin might be expected to counteract, at least in part, the growth retardation of immature rats maintained at low environmental temperatures. In the present communication data are presented on the effects of an anterior pituitary growth-promoting extract on the gain in body weight of immature rats under conditions of low environmental temperature.

PROCEDURE AND RESULTS

The diet employed in the present experiment consisted of sucrose, 60.0%; casein,[2] 25.0%; salt mixture,[3] 5.0%; and cottonseed oil (Wesson), 10.0%. To each kg. of the above were added the following synthetic vitamins: thiamine hydrochloride 40 mgms., riboflavin 40 mgms., pyridoxine hydrochloride 40 mgms., nicotinic acid 60 mgms., calcium pantothenate 80 mgms., 2-methyl-naphthoquinone 10 mgms. and choline chloride 2 g.[4] Each rat also received once weekly 4.5 mgm. of alpha-tocopherol acetate and a vitamin A-D concentrate containing 150 U.S.P. units of vitamin A and 15 U.S.P. units of vitamin D.[5] Forty-six male rats of the Long-Evans strain were selected at 24 to 27 days of age and an average weight of 57.2 g. for the present experiment. Tests were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of $2 \pm 1.5°$ C. and (2) under standard laboratory conditions at an average temperature of approximately $23 \pm 2°$ C. Group I consisted of 30 rats; group II of 16. Half the animals in each group received daily intraperitoneal injections of an anterior pituitary growth-

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[2] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[3] Hubbel, Mendel and Wakeman Salt Mixture, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] In view of the increased requirements for thiamine (Hegsted and McPhee, 1950; Ershoff, 1950) and pyridoxine (Gyorgy, 1938) under conditions of low environmental temperature in the rat, and B vitamins in the present experiment were administered in excessive amounts in order to assure an adequacy of these factors in the diet.

[5] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

promoting fraction[6] (1 mgm. per rat in 1 cc. of sterile saline solution); the other half served as untreated controls. Injections were continued for 18 days. The experiment was terminated 24 hours after the last injection. Food and water was provided *ad lib.*

Results are summarized in Table 1. Data in the cold room series were computed on the basis of the top 10 animals in each group in order to minimize variations in averages due

TABLE 1. EFFECTS OF "GROWTH HORMONE" ON THE GAIN IN BODY WEIGHT OF IMMATURE RATS UNDER COLD ROOM AND ROOM TEMPERATURE CONDITIONS

Group	Number of animals	Initial body wt.	Average gain in body wt. over 18 day period[1]
	Cold room series		
		g.	g.
Control	10	57.4	34.1 ± 2.4
Growth hormone	10	57.2	32.9 ± 4.3
	Room temperature series		
Control	8	56.9	66.7 ± 8.0
Growth hormone	8	57.3	85.0 ± 6.1

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma \frac{d^2}{n}} / \sqrt{n}$

where "d" is the deviation from the mean and "n" is the number of observations.

to early deaths, infection and atypical growth responses on the part of individual rats. Findings indicate that the gain in body weight of immature rats was markedly reduced under cold room conditions both in control and treated rats with no significant difference in the growth increment of the two groups. In the room tmperature series, however, gain in body weight was materially increased in the group receiving the pituitary material.

DISCUSSION

Present findings suggest that impaired formation or release of somatotrophin is not the cause of the growth retardation of immature rats under conditions of low environmental temperature. This is indicated by the fact that massive doses of a growth-promoting fraction of anterior pituitary did not counteract the retardation in growth or even augment the increment in body weight of immature rats under conditions of low environmental temperature. The pituitary material, however, did increase the gain in body weight of immature rats under room temperature conditions. Previous findings indicate that somatotrophin will not promote a weight increment in animals that are nutritionally deficient. Thus "growth hormone" failed to promote a gain in body weight in rats deficient in vitamin B_1 (Simpson and Evans, 1944) or vitamin A (Margitay-Becht and Wallner, 1937; Ershoff and Deuel, 1945) while similar results have been reported for rats fed a low protein diet (Gordan *et al.*, 1947). Results of the present experiment suggest that the environmental temperature is also of importance in determining

[6] We are indebted to Dr. I. M. Bunding of Armour and Co., Chicago, Ill., for the growth hormone employed in the present experiment. This material was a partially purified preparation (lot 22KR1) which when administered at a level of 50 gamma per day caused a weight increment of 1 g. per day in the 40–50 g. hypophysectomized rat.

the effects of somatotrophin administration in the rat since administration of a growth-promoting fraction of anterior pituitary increased the weight increment of immature rats under room temperature conditions but was without effect on the gain in body weight of immature rats under cold room conditions.

SUMMARY

1. Administration of massive doses of a growth-promoting fraction of anterior pituitary failed to counteract the growth retardation of immature rats under conditions of low environmental temperature.

2. A significant difference was observed in the effects of "growth hormone" administration between animals maintained under cold room (2° C.) and room temperature (23° C.) conditions. "Growth hormone" was without effect on the gain in body weight of immature rats under cold room conditions whereas it augmented the weight increment of animals maintained at room temperature.

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EFFECT OF ACTH ON THE INCORPORATION OF C^{14} OF GLYCINE IN TISSUE PROTEINS

White, Hoberman and Szego (1948) reported that the incorporation of N^{15} labelled glycine into tissues was more rapid in adrenalectomized than in normal animals. According to Clark (1950) in animals receiving cortisone there was a decrease in protein synthesis as compared to the controls. Friedberg (1947) observed that the administration of dehydrocorticosterone to adrenalectomized animals depressed the rate of incorporation of methionine sulfur (S^{35}) into tissue protein. It was suggested that when this hormone acted protein synthesis was slowed, amino acids accumulated, and hence, these acids were utilized at an increased rate for carbohydrate production.

If hypophysectomized rats are given ACTH there is a decrease in uptake of the C^{14} label of carboxyl marked glycine in the protein fraction as shown, e.g., for muscle and kidney.

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EFFECT OF ACTH ON THE INCORPORATION OF C^{14} IN TISSUE PROTEINS*

Tissue	Hypophysectomized animal	Hypophysectomized and ACTH treated animal
Muscle	2.24 ±0.3 (6)	0.97 ± 0.2 (6)
Kidney	76.0 ±5.6 (6)	48.0 ±14.0 (6)

The incorporation of C^{14} is expressed as counts/mg of protein/2 min. The arithmetic mean is given followed by the standard error of mean. Values in brackets indicate number of animals used.

* Supported by a grant from the Damon Runyon Memorial Fund.

EXPERIMENTAL

Male rats weighing 130–140 gm. were injected intramuscularly with 50 mg. ACTH (25% Armour Standard) distributed in 4 doses over 24 hours, two weeks after hypophysectomy. (The controls received purified egg albumin instead of ACTH.) Then, 2 mg. C^{14} carboxyl labelled glycine suspended in saline was given intraperitoneally. The animals were sacrificed 4 hours later and the tissues were analyzed as described by Friedberg and Webb (1949).

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USE OF OSMIUM TETRAOXIDE AS A REAGENT FOR ASSAYING CORTICOSTEROIDS

The present paper extends studies recorded in the literature concerning the use of osmium tetraoxide as a reagent for the detection of adrenal cortical steroids. Bennett has reported that filter paper impregnated with an alcoholic solution of 11-desoxycorticosterone acetate turns black when exposed to osmium tetraoxide. He also noted that the areas of black coloration obtained after osmium tetraoxide treatment of cat adrenal cortical tissue corresponded closely to areas which reacted with phenylhydrazine. These findings were interpreted to suggest that both reagents were reacting with steroids contained in the aderenal cortex.

The studies reported here were undertaken to determine the specificity of this osmium tetraoxide-corticosteroid reaction and hence its potential usefulness as a reagent for detecting corticosteroids in biologic fluids.

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This work was supported in part by grants from the Commonwealth Fund of New York and the American Cancer Society.

TABLE 1. TYPE OF OSMIUM TETRAOXIDE REACTION OBTAINED WITH MEASURED QUANTITIES OF VARIOUS SOLVENTS AND STEROIDS. RESULTS EXPRESSED AS POSITIVE, QUESTIONABLE OR NEGATIVE AS DESCRIBED IN TEXT

No.		Amount used
	Group A—Substances which react positively	μ
1	Corticosterone	3
2	17-OH corticosterone	3
3	11-dehydrocorticosterone	3
4	17-OH, 11 dehydrocorticosterone	3
5	11-desoxycorticosterone	3
6	11-desoxycorticosterone acetate	3
7	progesterone	3
8	testosterone	3
9	Δ4 androstenedione	3
10	cholestenone	3
	Group B—Substances which react questionably	
11	pregenolone	100
12	acetoxypregnenolone	100
13	ethinyltestosterone	100
14	androstenediol-3-17-acetate	100
15	dehydroisoandrosterone	200 to 500
16	3-chlorodehydroisoandrosterone	250
	Group C—Substances which do not react	
17	pregnanediol	500
18	cholesterol	300
19	cholestanone	250
20	cholestanol	250
21	androsterone	500
22	isoandrosterone	200
23	etiocholanone	100
24	estrone	500
25	estriol	250
26	estradiol	500
27	estradiol benzoate	500
28	dextrose	500
		cc.
29	water	1
30	methanol, absolute	1
31	ethanol, absolute	1
32	ethyl ether	1
33	acetone	1
34	propylene glycol	.05
35	benzene	1
36	toluene	1
37	chloroform	1
38	carbon tetrachloride	5

METHODS OF PROCEDURE

Solvents to be tested were impregnated on to the entire surface of a 1×5 cm. strip of ethyl ether washed Whatman No. 1 or 2 filter paper. Steroids to be tested were dissolved in absolute methanol at a known concentration. A measured amount of this solution, usually 0.05 cc., was placed within a circular area 6 mm. in diameter in the center of a similar strip of filter paper with the aid of fine tipped, calibrated 0.2 cc. pipette. During impregnation the paper strip was held in air by means of chemically clean, small

caliber forceps. Each impregnated strip was identified by cutting off one or more corners or by other variations in shape.

After impregnation, the strip was suspended in a stream of air at a temperature not exceeding 40° C. until dry. It was then suspended by an ethyl ether washed thread several centimeters above the bottom of a tightly stoppered, dark colored glass jar containing about 10 cc. of a freshly prepared, 1 per cent aqueous solution of osmium tetraoxide. Care was taken to prevent the paper strips from coming in direct contact with this solution or with each other. After 24 hours exposure to the osmium tetraoxide vapors at room temperature, the paper strips were removed. In early studies the strips were washed for 24 hours in a stream of tap water, then dipped in 80 per cent aqueous ethanol solution and finally allowed to dry. Later these steps were found to be unnecessary.

RESULTS

The types of reaction observed were arbitrarily divided into three types: negative (no color), questionable (faint brown) and positive (definite grey-black). Table 1 indicates the reactions obtained with the various substances tested. It is seen that all solvents (group C) were negative in the quantities tested. Tests performed on methanol solutions of the non-volatile residue remaining after distillation of larger volumes of certain of these solvents have given questionable or positive reactions. However, only negative reactions were had with the non-volatile residues remaining after evaporation of the mid-fraction of solvent distillates. These findings suggest that the solvents listed may contain non-volatile impurities which react with osmium tetraoxide to yield a brown or black pigment.

The steroids investigated appeared to be divisible into three groups, namely those which reacted positively in 3 microgram quantities (group A), those which gave a questionable faint brownish color (group B) when 100 or more microgram quantities were tested and those which failed to react in amounts ranging between 100 and 500 micrograms (group C). Positive reactions became discernible within 15 minutes after exposure of the steroid to osmium tetraoxide vapors. The intensity of the reaction observed for group A substances appeared to bear a direct and approximately quantitative relation to the amount of substance tested.

COMMENTS

Inspection of the information presented in Table 1 reveals that the steroids giving a positive reaction with osmium tetraoxide have as a common characteristic a $\Delta 4$, 3 ketone configuration. Except for substance 13, compounds yielding a questionable reaction lack this characteristic. Interestingly, a number of them (substances No. 11, 12, 14 to 16) have either a $\Delta 5$, 3 hydroxyl or chloro group. Steroids which failed to react are seen to be either saturated 3-ketonic or 3-hydroxy steroids or phenolic estrogens.

The foregoing information, coupled with the observation that dextrose and various solvents fail to give a positive osmium tetraoxide reaction, suggest that this reaction may be of value in the detection and characterization of steroids extracted from biologic fluids. Preliminary studies already have shown that an approximate idea of the urinary corticosteroid output may be had by application of this procedure to the residue of sodium hydroxide and water washed chloroform extracts of human urine. The reaction also appears of potential value as a means for revealing the position of urinary corticosteroids following paper chromatography of purified urinary extract material.

SUMMARY

Studies made with various crystalline steroids show that osmium tetraoxide reacts quite specifically with steroids having as a common characteristic an alpha-beta unsaturated 3-ketone group. This observation suggests that the reaction may be of value in the detection and characterization of steroids in extracts of biologic fluids.

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REFERENCE

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The authors are indebted to Dr. E. C. Kendall of the Mayo Clinic for supplies of crystalline corticosteroids, to Dr. E. Schwenck of the Schering Corporation for supplies of other steroids and to Dr. N. B. Talbot for advice in the preparation of this paper.

[1] John Simon Guggenheim Fellow.

THE EFFECT OF ALLOXAN IN METHIONINE- AND CYSTINE-DEFICIENT GUINEA PIGS

Collins-Williams, Renold, and Marble (1950) reported that guinea pigs fed a methionine- and cystine-deficient diet for 3–4 weeks develop spontaneous hypoglycemia 4 hours after removal of food. They state that the effect of fasting on methionine- and cystine-deficient guinea pigs was not indicated by Griffiths (1948a) who found that alloxan caused hypoglycemia in these guinea pigs. Nevertheless it was stated that all animals were fasted 17 hours. From the figures given it is apparent that the average fasting blood sugar value of the methionine- and cystine-deficient guinea pigs was 120 mg. per cent.

Collins-Williams *et al.* found an extraordinary high death rate among their guinea pigs fed the deficient diet. This suggests that the few survivors were moribund rather than simply cystine deficient. Apparently moribund animals become hypoglycemic when food is removed for a short time.

An explanation which possibly explains the discrepancy in results is that the diet fed by Collins-Williams *et al.* was a modification of the Haag and Wright (1940) diet described by Griffiths (1948b, 1950) as effective in lowering the reduced glutathione (GSH) in the blood of rabbits. The diet used by Griffiths in the guinea pig experiments was stated to be the Haag and Wright diet plus 0.1 per cent ascorbic acid. The modified diet used by Collins-Williams *et al.* contained half the protein of the Haag and Wright diet and synthetic B group vitamins instead of a supplement of yeast.

A further series of experiments have been carried out in which 17 adult guinea pigs were fed the Haag and Wright diet plus 0.1 per cent ascorbic acid for 24 days.[1]

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[1] It may be pertinent to mention that, as a matter of routine, all animals were fed a complete laboratory diet and fresh greens daily for at least two months before they were used for experiments.

The animals were housed in a room thermostated at 70° F. During the 24-day period, four animals died.

After a 17 hour fast six animals received 600 mg./Kg. alloxan monohydrate (BDH product) intraperitoneally and the remaining seven received an intraperitoneal injection of water. At the same time six normal guinea pigs were injected in the same manner with 600 mg./Kg. alloxan monohydrate. The average fasting blood sugar values of the three groups were 121, 129 and 97 mg. per cent respectively. The result of the alloxan injections in the cystine-deficient guinea pigs was death, severe hypoglycemia, or no effect at all, within 7 hours of injection. The average blood sugar value of this group was 72 mg. per cent and that of the control group (which was outwardly unaffected) was 117 mg. per cent. The difference between these two means was significant with a value of p greater than .02, whilst the difference between the means 121 and 129 was not significant. The normal animals were resistant to the alloxan and showed no change in blood sugar level at 7 hours after injection (average blood sugar value of 105 mg. per cent).

It was considered possible (Griffiths 1948a) on the basis of Lazarow's (1946) experiments that the resistance of guinea pigs to alloxan was due to their relatively high blood GSH. This possibility is further substantiated by the results of some determinations of blood GSH by the method of Woodward and Fry (1932),[2] in the common fowl Gallus domesticus which is extremely resistant to the diabetogenic effect of alloxan (Scott, Harris and Chen 1945). It was found that the average blood GSH of a group of six roosters was 60 mg. per cent (range 55–65) which is in good agreement with the average figure of 58 mg. per cent for guinea pigs. Both these values are considerably higher than the values of 35–40 mg. per cent which are consistently found in rabbits in this laboratory.

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[2] As the uric acid metabolism of birds is considerably different to that of mammals the effect of uric acid on glutathione estimations was determined and was found to be nil.

The amount of sulphosalicylic acid needed to precipitate rooster blood proteins was double that sufficient for mammals. The method was calibrated against pure glutathione using the increased amount of sulphosalicylic acid.

BOOK REVIEWS

One of the interesting developments in medical publication is the recent series of small monographs undertaken by Mr. Charles C Thomas, Publisher, Springfield, Illinois. In these monographs, current topics are presented without the usual limits of space or time ordinarily imposed by publications such as the Annual Reviews. The list is an actively growing one. So far, a number of issues are available which are of interest to Endocrinologists. Among these are the following:

The clinical use of testosterone, 69 pp. by Henry H. Turner

On the experimental morphology of the adrenal cortex, 105 pp. by Hans Selye and Helen Stone

Electrophoresis in physiology, 66 pp. by Lena A. Lewis

Chemical developments in thyroidology, 87 pp. by William T. Salter

Primary carcinoma of the vagina, 73 pp. by Robert G. Livingstone

Enzymes, growth and cancer, 64 pp. by Van R. Potter

Simmond's disease, Extreme insufficiency of the adenohypophysis, 93 pp. by R. F. Farquharson

Obesity, 134 pp. by Edward H. Rynearson and Clifford F. Gastineau

Diagnosis and therapy of gynecological endocrine disorders, 59 pp. by C. L. Buxton and E. T. Engle

The renal origin of hypertension, 126 pp. by Harry Goldblatt

The diagnosis and treatment of adrenal insufficiency, 180 pp. by George M. Thorn.

E.W.D.

ASSOCIATION NOTICES

To the Editor of the *Journal of Endocrinology*

Dear Sir:

Through you I should like to discuss with your readers, and particularly with the members of the Association for the Study of Internal Secretions, the type of program which we are planning for Atlantic City in June of 1951.

At our meeting in San Francisco we voted to have three days of scientific programs, rather than the two we have had in the past. Dr. Robert Williams, of Seattle, Washington, wrote and suggested that we try grouping the papers under separate headings, and this suggestion met with favor in the Council meeting.

Accordingly, we have made arrangements for Thursday, Friday and Saturday, June 7th, 8th, and 9th, the meetings to be held either in the Chalfonte or Haddon Hall hotels. Tentatively, we are suggesting the following schedule:

THURSDAY: 9:30 A.M.—General subjects.
2:00 P.M.—Papers relating to the pituitary body.
8:00 P.M.—"Information Please," a program of informal discussion with the audience and a "Board of Experts" taking part.

FRIDAY: 9:00 A.M.—Papers relating to the thyroid and parathyroid glands.
2:00 P.M.—Papers relating to the adrenal glands.
Evening —The annual cocktail party and banquet.

SATURDAY: 9:00 A.M.—Papers relating to gonads and sterility.
2:00 P.M.—Joint session with the American Diabetes Association.

For each of these sessions there will be two presiding officers; one interested principally in clinical medicine, the other in experimental studies. These doctors will be responsible for the selection of papers for their own program, the title and abstracts being sent to them by the chairman of the Program Committee.

This letter is accompanied by instructions for the submission of abstracts. These instructions were prepared by Dr. Edward Doisy. Last year he and his associates and their secretaries had several weeks of needless work due to the incompleteness of information. Dr. Jane Russell, School of Medicine, Emory University, Georgia, is chairman of the Program Committee for 1951. We are particularly desirous of having more papers relating to clinical medicine submitted for these programs. The early submission of titles and complete abstracts will greatly facilitate planning of our meeting. Because so many members of the Program Com-

mittee will need to review these abstracts, we must continue to ask for four copies. No abstracts received after March 1, 1951, can be considered.

We are going to try this three-day meeting for 1951. The course to be followed in subsequent years will be determined by vote at the annual meeting at the conclusion of this program.

THE 1951 AWARDS OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

FELLOWSHIPS

The Ayerst, McKenna and Harrison Fellowship

The Ayerst, McKenna and Harrison Fellowship was first awarded in 1947 to Dr. Samuel Dvoskin and in 1948 to Dr. Ernest M. Brown, Jr. Dr. Brown was re-elected for the Fellowship in 1949, and the 1950 Fellowship was awarded to Dr. Lawrence E. Shulman.

The Schering Fellowship in Endocrinology

The Schering Fellowship in Endocrinology was established in 1949 and the first recipient was Dr. D. Laurence Wilson, who also received the 1950 Fellowship.

Association Fellowships are designed to assist men or women of exceptional promise in their progress toward a scientific career in endocrinology. Fellowships may be awarded to an individual who possesses the Ph.D. or M.D. degree or to a candidate for either of these degrees. The stipend, which will not exceed $2,500.00, may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointee. The Committee will, in reviewing the proposed program of study, consider the amount of time which the Fellow intends to spend in course work and/or teaching. He must present evidence of scientific ability as attested by studies completed or in progress and/or the recommendation of responsible individuals. He must submit a program of proposed study, indicate one or more institutions where the proposed program will be followed, and submit statements of approval from the investigators with whom he proposes to conduct his research. He must serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be allotted for course work or for participation in teaching, the latter purely on a voluntary basis.

AWARDS

The E. R. Squibb and Sons Award

The E. R. Squibb & Sons Award of $1,000.00 was established in 1929, and was given first in 1940 to Dr. George W. Corner; in 1941 to Dr.

Philip E. Smith; in 1942 to Dr. Fred C. Koch; in 1944 to Dr. E. A. Doisy; in 1945 to Dr. E. C. Kendall; in 1946 to Dr. Carl G. Hartman; in 1947 to Drs. Carl F. and Gerty T. Cori; in 1948 to Dr. Fuller Albright; in 1949 to Dr. Herbert M. Evans; and in 1950 to Dr. C. N. H. Long. In 1943 no award was given. A special committee of five members of the Association selects the recipient from among investigators in the United States or Canada, on the basis of outstanding contributions to endocrinology.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishment of an investigator not more than thirty-five years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the award was presented to Dr. E. B. Astwood; in 1945 to Dr. Jane A. Russell; in 1946 to Dr. Martin M. Hoffman; in 1947 to Dr. Choh Hao Li; in 1948 to Dr. Carl Heller; in 1949 to Dr. George Sayers; and in 1950 to Dr. Oscar M. Hechter. The Award is for $1,200.00. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $1,800.00.

NOMINATIONS

Each member has the privilege of making one nomination for each Fellowship or Award. A nomination should be accompanied by a statement of the importance of the nominee's contributions to, or interest in, endocrinology and by a bibliography of the nominee's most important publications, with reprints if possible. The nominations should be made on special application forms which may be obtained from the Secretary, Dr. Henry H. Turner, 1200 North Walker Street, Oklahoma City 3, Oklahoma, and returned to him not later than March 15, 1951.

THE 1951 MEETING OF THE ASSOCIATION FOR THE STUDY OF INTERNAL SECRETIONS

The Thirty-third Annual Meeting of the Association for the Study of Internal Secretions will be held at the Chalfonte-Haddon Hall Hotel, Atlantic City, New Jersey. The Scientific Sessions have been extended to three days, Thursday, Friday, and Saturday, June 7-8-9.

The Local Chairman of Arrangements is Dr. Matthew Molitch.

Due to the advance in the date of our meeting, Chalfonte-Haddon Hall Hotel will be unable to accommodate all of our members by reason of a previous commitment. The Colton Manor, on Pennsylvania Avenue near Haddon Hall, is an excellent hotel, which has been recently refurnished, and is holding one hundred rooms for our members on the "first come, first served" basis. The Hotel Strand, at Pennsylvania Avenue and the Boardwalk, has also promised to supply a limited number of rooms. It is important that reservations be made immediately if you are to avoid disappointment.

All Scientific Sessions will be held in the Chalfonte-Haddon Hall Hotel. The rooms, in which the session will be held, will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, June 8, at 7:30 P.M. in the Rutland Room, preceded by cocktails at 6:30 P.M. in the adjoining Benjamin West Room.

Those wishing to present papers, which will be limited to ten minutes, should send four copies of the title and abstract to the Vice-President, Dr. Jane Russell, School of Medicine, Emory University, Atlanta, Georgia, not later than March 1, 1951. *It is imperative that the abstracts be informative and complete with results and conclusions in order that they may be of reference value and suitable for printing on the program and journals of the Association.* The following regulations for the preparation of abstracts and titles must be carefully followed to insure consideration of the paper for the program.

1. Abstracts may not exceed two hundred words, or equivalent space, exclusive of title. No footnotes or acknowledgments to sponsors can be published. References, if used, must be placed in the body of the text. The abstract should consist of a single paragraph, if possible. Structural chemical formulas cannot be used.
2. The title heading must be arranged as follows:
   Line 1. Title, not to exceed fifteen words.
   Line 2. Author/s. The name of each non-member author collaborating with member-authors is to be followed by the phrase "(by invitation)." Names of non-members who are introduced, i.e., who are not collaborators with member-authors, are to be followed by the phrase "(introduced by . . .)." The principal degree, i.e., M.D., of each author should be given after his name.
   Line 3. Institution of origin; city in which institution is located.

3. The body of the abstract, typed double space, should follow the heading. The original copy should be on bond paper. There should be three copies.
4. Abstracts should be letter-perfect, since there will be no opportunity for proof reading by authors.

Nominations for the Ayerst, McKenna and Harrison Fellowship, the Schering Fellowship in Endocrinology, and the Squibb and Ciba Awards should be made on special application forms, which may be obtained from the Secretary-Treasurer, Henry H. Turner, M.D., 1200 North Walker Street, Oklahoma City 3, Oklahoma, and filed with the Secretary not later than March 15, 1951.

EDWARD H. RYNEARSON, *President*